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## **Investigating breast cancer O-linked glycosylation changes and their immunotherapeutic potential**

Picco, Gianfranco

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***Investigating breast cancer O-linked glycosylation changes and their immunotherapeutic potential.***

PhD in Medicine, 2013, Gianfranco Picco.

King's College London

## ***Abstract***

Changes that occur in malignant cells can take a variety of forms and include changes in cellular glycosylation. Aberrant glycosylation is considered to be a universal feature of malignant transformation and tumour progression. Mucins are major carriers of altered glycans in most carcinomas, and mainly belong to the MUC family, with MUC1 considered one of its most prestigious affiliates, as it is upregulated and aberrantly glycosylated in the majority of breast and many other adenocarcinomas. The large domain of the extracellular region of MUC1 is made up of tandemly repeated amino acids that form a scaffold for the attachment of the O-linked glycans. The aberrant glycosylation of MUC1 observed in breast cancer results in the expression of simple, unbranched sugars that are often sialylated and this is partly due to the upregulation of the sialyltransferase, ST3Gal-I. These consistent and conserved changes suggest that aberrant glycosylation is advantageous for the tumour, and data generated by this thesis suggest that indeed this is the case.

Attention has been focused on MUC1 as a target molecule for the immunotherapy of cancer. To investigate specific MUC1 tumour-associated glycans, recombinant MUC1 glycoproteins carrying specific and defined O-linked glycans were purified from engineered CHO cells. The immune responses to these MUC1 glycoforms were analysed using wild-type and MUC1 transgenic mice. Different tumour-associated glycoforms elicited different immune responses, with MUC1 carrying the disaccharide known as T inducing a cellular response in MUC1 Tg mice, while the MUC1-ST glycoform was incapable of inducing an immune response in MUC1 Tg mice or even wild-type mice.

To determine whether the changes in glycosylation, observed in breast carcinomas, confer an advantage to cancer cells, we generated transgenic mice over-expressing ST3Gal-I in the mammary gland and other epithelial tissues, and crossed these with mice that develop spontaneous mammary tumours. The tumours that developed from the ST3Gal-I mice appeared significantly earlier than the controls. Thus over-expression of ST3Gal-I in the mammary gland promotes tumorigenesis in this model, and the MUC1-ST glycoform formed by the action of ST3Gal-I on the T antigen is not immunogenic and may even inhibit an immune response.



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## ***Abbreviations***

4B10: Anti human ST3Gal-I antibody  
5E5: Anti-MUC1-Tn/STn antibody  
ADCC: Antibody-dependent cellular cytotoxicity  
AKT: Serine/threonine kinase  
APC: Antigen presenting cell  
BSA: Bovine serum albumin  
CDGS: Carbohydrate-deficient glycoprotein syndromes  
CFG: Consortium of Functional Glycomics  
CHO: Chinese hamster ovary cell line  
CHO K1: Chinese hamster ovary cell line (parental line)  
CIS: Carcinoma *in situ*  
CNA: Copy number aberration  
CNS: Central nervous system  
COX-2: Cyclooxygenase-2  
CPM: Counts per minutes  
CT: Cytoplasmic tail  
CTL: Cytotoxic T lymphocyte  
DCIS: Ductal carcinoma *in situ*  
DGC: Dystrophin-glycoprotein complex  
DNA: Deoxyribose nucleic acid  
EDTA: Ethylenediaminetetraacetic acid  
EGF: Epidermal growth factor  
EGFR: Epidermal growth factor receptor  
ELISA: Enzyme-linked immunosorbent assay  
EMA: Epithelial membrane antigen  
EPO: Erythropoietin  
ER: Oestrogen receptor  
GAGs: Glycosaminoglycans  
Gal: Galactose  
GalNAc: N-acetylgalactosamine  
GI: Gastro intestinal

GlcNAc: N-acetylglucosamine  
 GSLs: Glycosphingolipids  
 GVHD: Graft-versus-host disease  
 HER2: ErbB2  
 HMFG1: anti-MUC1 antibody  
 HMFG2: anti-MUC1 antibody  
 IDO: Indoleamine 2,3-dioxygenase  
 IEX: Ion exchange column  
 ITIM: Immunoreceptor tyrosine-based inhibitory motif  
 LPS: Lipopolysaccharide  
 MAA: Maackia amurensis lectin  
 MAGE: Melanoma antigen-encoding gene  
 MCF7: Human breast adenocarcinoma cancer cell line  
 MDSCs: Myeloid-derived suppressor cells  
 MFI: Mean fluorescence intensity  
 MGL: Macrophage galactose/N-acetylglactosamine-specific lectin.  
 MHC: Major histocompatibility complex  
 MUC1: Mucin 1  
 Muc1: Mucin 1 (mouse)  
 MUC1-CT: Cytoplasmic tail of MUC1  
 MUC1\*: Post cleavage, transmembrane MUC1  
 N-linked glycan: N-(Asn)-linked glycan  
 Neu5Ac: Sialic acid  
 NK: Natural killer  
 O-linked glycan: O-(Ser/Thr)-linked glycan  
 PAMP: Pathogen associated molecular pattern  
 PBS: Phosphate buffered saline  
 PF: pathogen free  
 PGE2: prostaglandin E2  
 PGs: Proteoglycans  
 PyMT: Polyoma middle T  
 PNA: Peanut agglutinin lectin  
 PolyIC: Polyinosinic:polycytidylic acid  
 PR: Progesterone receptor

PTM: Post-translational modification  
rhMUC1: Recombinant human MUC1  
RNA: Ribose nucleic acid  
RT: Room temperature  
SM3: anti-MUC1 antibody  
SPF: Specific pathogen free  
STAT: Signal transducers and activators of transcription  
T47D: Human ductal breast epithelial cancer cell line  
TAA: Tumour associated antigen  
TBS: Tris buffered saline  
TCR: T cell receptor  
TGF-  $\beta$ 1: tumour growth factor  $\beta$ 1  
Th1: Differentiated CD4 cell associated with acute inflammation and a CTL response  
Th17: Differentiated CD4 cell associated with chronic inflammation  
Th2: Differentiated CD4 cell associated with a humoral response  
TNF- $\alpha$ : Tumour necrosis factor  $\alpha$   
TNM: Tumour node metastases  
TKH2: Anti-STn antibody  
TLR: Toll-like receptor  
Treg: Regulatory T cell  
TRP2: Tyrosinase-related protein2  
TSA: Tumour specific antigen  
VAGF: Vascular endothelial growth factor  
VNTR: Variable number of tandem repeats

# ***Chapter 1***

## ***Introduction***

## 1.1 General Introduction

Glycobiology, the study of structure, biosynthesis and biology of saccharides (sugar chains or glycans) is one of the most rapidly growing fields in biomedical sciences, with relevance in basic research, biomedicine and biotechnology. Cells carry a dense and complex array of sugar chains, and since most of the glycans are on the outer surface of cells, they are in a position to modulate or mediate many cell interactions crucial to the development and function of complex multicellular organisms, but also interactions between organisms such as host to pathogens.

The glycosylation found in eukaryotic cells is primarily defined according to the nature of the linkage to the protein or lipid. Two of the most common types of protein glycosylation are N-linked glycans (N-(Asn)-linked glycans) and mucin type O-linked glycans (O-(Ser/Thr)-linked glycans). N-linked glycans are covalently linked to an asparagine residue of a polypeptide chain, while mucin type O-linked glycans are linked via N-acetylgalactosamine (GalNAc) to a serine or threonine residue and can be extended into a variety of different core structures (Varki A. *et al.* 2009). Unlike N-linked glycans, which are initially added *en bloc*, O-linked glycans are added individually and sequentially, with each linkage being catalysed by a specific glycosyltransferase.

In cancer, tumour cells undergo activation and division, adhere to a variety of other cell types and cell matrices and invade tissues. To some extent this parallels patterns and processes observed during embryogenesis. More than 90% of cancer cases are attributed to environmental factors such as environmental pollutants, diet and radiation, and 5-10% of cases are due to inherited genetic mutation (Anand P. *et al.* 2008). Genes in cancer cells can be modified by mutations, which can inhibit expression or alter the function of the protein they encode, or through epigenetic modifications to chromatin, which alter the gene-expression pattern.

The changes that occur in malignant cells can take a variety of forms, for example loss of expression or increased expression of certain proteins. However, changes in cellular glycosylation are a universal feature of malignant transformation and tumour

progression (Ono M. and Hakomori S. 2004). For example, cancer cells are generally found to have an increase in  $\beta$ 1-6 branching N-linked glycans (Varki A. *et al.* 2009), and the composition and number of O-glycans added to cell surface mucins is also altered (Muller S. *et al.* 1999, Burchell J. M. *et al.* 2001, Mungul A. *et al.* 2004).

## ***Aims***

This thesis focuses on breast cancer and the associated changes that occur in mucin O-linked glycosylation, with relevance to the immune system. Some of the most striking glycosylation changes in cancer occur to the O-glycans carried by mucins, so the focus has been on these changes, and in particular those on human mucin 1 (MUC1), one of the major and most abundant mucins aberrantly glycosylated in breast cancer. The relevance of O-linked glycosylation to changes in tumour growth and the effects on interactions with the immune system has been examined. The exploitation of MUC1 glycosylation changes as a target for immunotherapeutic approaches has also been investigated.

The aims of this project were to analyse the immune response to various glycoforms of MUC1 and investigate the mechanism involved in that response. MUC1 carrying specific and defined O-linked glycans has been expressed in CHO cells and CHO cell derivatives and purified. The immune response to these glycoforms was analysed, *in vitro* and *in vivo* using wild-type and MUC1 transgenic mice. Also, in order to determine if changes in glycosylation observed in breast carcinomas confer an advantage to cancer cells, transgenic mice over-expressing a breast cancer associated glycosyltransferase were generated, and crossed with mice that develop spontaneous mammary tumours. The growth rate of these tumours was observed and possible correlations investigated.

## 1.2 Breast cancer

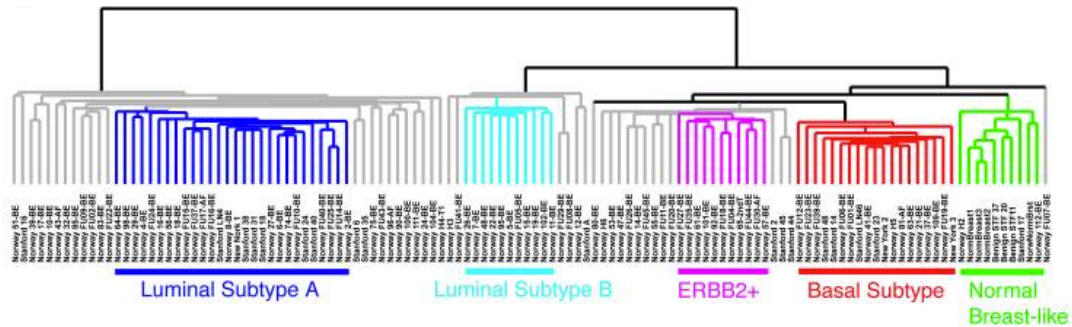
With over 40,000 new cases every year in the UK, breast cancer is the most common of all malignancies to affect women. The incidence has risen over the past 50 years and globally the incidence is approximately 1 million new cases per year, making it the 3rd most common cancer in the world, with approximately 400,000 deaths each year (Tobias J. and Hochhauser D. 2010). It is estimated that 1100 people die of breast cancer per day across the world (Parkin D. M. 2001) and that in the UK about 1 in every 9 women will develop breast cancer at some point during their lives.

The vast majority of breast cancers are adenocarcinomas, arising from glandular epithelial cells in the breast ducts or lobules. On presentation, most cancers have invaded the stroma and are therefore locally invasive. However, some women present with tumours that have not invaded the basement membrane and are termed CIS (carcinoma *in situ*), with the most common form being ductal carcinoma *in situ* (DCIS). Early breast cancer is often asymptomatic, and patients may find it difficult to identify, as breast tissue is naturally lobular and changeable. However, in recent years, with the introduction of mammography screening, the number of DCIS cases detected has increased and around 20% of breast cancer patients now present with DCIS (Ernster V. L. *et al.* 2002).

Recent advances in genetic and proteomic profiling have contributed to a better understanding of breast cancer. Gene expression analysis has shown that it is possible to identify specific patterns, or signatures, which relate not only to phenotype but also to prognosis.

In 2001 Sorlie and colleagues proposed a classification system based on expression analysis, which identified sub-groups of breast cancer, which correlated with histological markers (fig 1.1) (Sorlie T. *et al.* 2001). When this classification was applied to survival, diversity of outcomes became apparent. The luminal A subgroup (representing 45% of all breast cancers), have a remarkably good prognosis (about 90% survival after 8 years), whereas the basal group, which represent 15% of breast cancers patients, have a poor prognosis, with the vast majority (about 70%) of patients assessed deceased within 4 years. This diversity of outcome highlighted the

importance of defining the subclass of breast cancer to design more appropriate treatments.

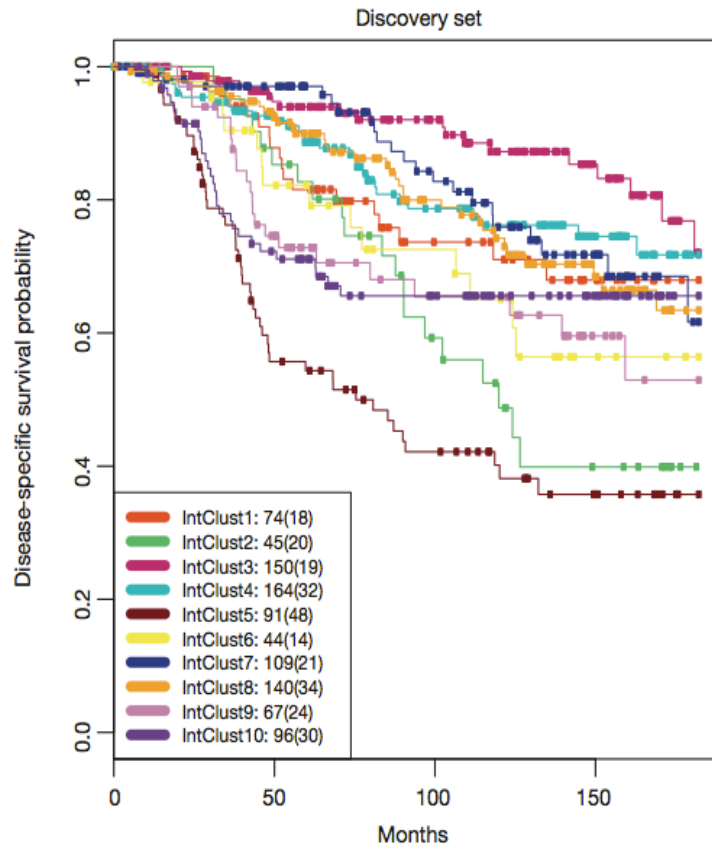


**Figure 1.1**      *Gene expression patterns of 85 breast cancers.*

*Tumour specimens were divided into five subtypes based on differences in gene expression. The diagram show five subtypes of tumours coloured as: luminal subtype A, dark blue; luminal subtype B, light blue; ERBB2+, pink; basal-like, red; and normal breast-like, green. (Taken from Sorlie T. et al. 2001)*

More recently, a multicentre team of investigators, which included members of our department, investigated the genomic and transcriptomic architecture of about 2000 breast cancer tumours (Curtis C. *et al.* 2012). This revealed novel subgroups with distinct clinical outcomes. The tumours were analysed by joint clustering of copy number and gene expression data, dominated by *cis*-acting copy number aberrations (CNAs). Ten clusters of breast cancer were identified (fig 1.2). Importantly, this analysis revealed a subgroup of oestrogen receptor positive tumours (ER+) that, in contrast to the majority of ER+ tumours, had a very poor prognosis (cluster 2, green line in fig 1.2), suggesting that additional therapy may be required for these patients.





*Figure 1.2 Copy number aberration grouping relation to clinical outcome of breast cancer patients.*

*Kaplan-Maier plot of disease-specific survival, truncated at 15 years, generated by grouping well define copy number aberrations (Taken from Curtis C. et al. 2012).*

Breast cancer can spread and disseminate quickly within the mammary glands and other organs, being so closely situated to the well-connected lymphatic system. The growth of the vascular and lymphatic network “angiogenesis” is very important for the growth of tumours and the metastatic spread of cancer tissue (Gelao L. *et al.* 2013). Lymph-borne secondaries mainly occur in the supraclavicular and axillary nodes, but can spread to other regional nodes, as well as the chest wall and its structures. Metastases can also localise to the bone, liver, lung, skin and CNS as secondary or tertiary sites. However, in some cases the disseminated cells may remain dormant for many years before metastases occur. It is this secondary disease that invariably causes the death of the patient, and there is a strong inverse relationship between the number of metastatic lymph node lesions and survival (Tobias J. and Hochhauser D. 2010).

Evidence is now accumulating that glycans play a role in dissemination and determining the destination of metastatic cells (Barthel S. R. *et al.* 2009; Julien S. *et al.* 2011). In a recent study from our group, in which I collaborated, oestrogen receptor alpha-negative (ER-) tumours expressed increased levels of sialyl-Lewis x (sLe<sup>x</sup>) as compared to ER positive. SLe<sup>x</sup> expression *per se* had no influence on the survival however high expression was significantly associated with bone metastasis. Interestingly, this was seen only in oestrogen receptor (ER) positive cancer, suggesting that the backbone on which the glycan is carried is important for its involvement in the extravasation process required for metastatic spread (Julien S. *et al.* 2011).

### ***1.2.1 Current treatment of breast cancer***

Breast cancer is usually treated according to the stage of the disease at presentation (TNM; tumour, node, metastases), the grade (measure of differentiation), the cancer type (luminal invasive, basal invasive, DCIS), histological markers (ER, PR and HER2) and the patient's menopausal status and general health (Tobias J. and Hochhauser D. 2010). Generally, healthy, late stage, high grade, triple negative patients receive the most aggressive treatments.

Standard treatments include surgery, radiotherapy, hormone therapy, chemotherapy, immunotherapy and targeted therapy. Surgery is usually the first treatment, with a fine balance being drawn between removing the lesion with a good margin, and the cosmetic and psychological impact on the patient. Preoperative chemotherapy is increasingly becoming common practice as neo-adjuvant therapy where a large tumour is shrunk before surgery to allow for more breast tissue conservation. Following surgery the vast majority of patients (90-95%) go on to receive some sort of adjuvant therapy, in the form of radiotherapy, hormone therapy, chemotherapy, immunotherapy or targeted therapy to inhibit growth of disseminated tumour cells (Turner N. C. and Jones A. L. 2008). Furthermore, when physicians make judgements as to which extra or adjuvant therapies to include, age, menopausal status, tumour grade, tumour burden, lymph node involvement, and expression of receptors on tumour such as HER2 are taken into consideration.

### ***Radiotherapy***

Classically radiotherapy will follow surgery as it is a localised treatment, most effective on smaller lesions or post-surgical ‘clean-up’, and, in some cases it is used as neo-adjuvant therapy. The use of neo-adjuvant radiotherapy can influence the amount of tissue removed, with many women choosing minimal surgery with maximum radiotherapy. Radiotherapy is a targeted high-energy beam that causes the cells to undergo apoptosis and die. Advances in radiotherapy machines and targeting, such as its use in combination with CT scanning, mean that the risk of more severe side effects such as excessive skin burning and damage to the heart and lungs can be greatly reduced (Edlund T. and Gannett D. 1999). Breaking the dose into fractions can also reduce side effects, which can still be substantial. Tiredness, followed by skin redness and burn are the most common side effects, along with lymphoedema, and some minor effects that are more cosmetic than troublesome, such as skin darkening. However, the major drawback of radiotherapy is that it is limited to use around the area of the primary tumour and is not a systemic treatment. For patients with no lymph node involvement, i.e. no evidence of metastasis, this is not a problem, but where tumour cells have moved elsewhere in the body, a more widespread treatment, such as chemotherapy is usually needed.

### ***Chemotherapy***

Chemotherapy is also designed to cause apoptosis in cells, but acts by disrupting the cell’s basic functions for example the generation and repair of the actin tubules and other internal structures involved in cell stability and division. Because tumour cells divide more rapidly than many normal cells they are preferentially affected. In addition, it has recently been shown that some chemotherapeutic agents have immune-stimulatory and anti-angiogenic properties (Liu W. M. *et al.* 2010, and 2012). In some cases chemotherapy is used as neo-adjuvant therapy to shrink the tumour thereby allowing more breast-conserving surgery. Whether or not they have received neo-adjuvant chemotherapy, the vast majority of patients will receive the treatment postoperatively to kill any cancer cells that may not have been removed through surgery. Side effects include nausea, vomiting and hair loss, as well as increased risk of infection and anaemia, and are brought about by damaging normal cells that have a high proliferative rate such as hair follicles, bone marrow and

haemopoietic cells. In the longer term, gonads may become damaged enough to cause infertility and early menopause.

### ***Endocrine therapy***

Hormone therapy may also be given as adjuvant treatment to women whose tumours are ER-positive, although sometimes hormone therapy can also be used in a neo-adjuvant setting. The oestrogen receptors, when engaged, can enhance the growth of the tumour, and hormone therapy is designed to prevent the supply of oestrogen to tumour cells. Drugs such as Tamoxifen block the binding of oestrogen to the oestrogen receptor, preventing any engagement of any oestrogen present in the system resulting in tumour growth inhibition. The oestrogen receptor/Tamoxifen complex can bind to different co-factors, including co-repressors that inhibit ER $\alpha$  mediated transcription (Green K. A. and Carroll J. S. 2007). However, with time many patients become resistant to Tamoxifen (Thrane S. *et al.* 2013). Recent studies have established that Tamoxifen is extensively metabolized by the enzyme CYP2D6. Patients who carry a particular polymorphism in the gene encoding the CYP2D6 enzyme have a lower response to Tamoxifen. This is because certain polymorphisms result in a lack of enzyme activity, and therefore these patients lack the ability to produce endoxifen, which is necessary for successful treatment (Irvin W. J. *et al.* 2011, Binkhorst L. *et al.* 2012). Aromatase inhibitors may also be used in postmenopausal women, which act by preventing the conversion of androgen to oestrogen by aromatases in the fatty tissue, thus limiting the supply of oestrogen in the circulation.

Side effects of endocrine therapy vary. Endocrine therapy can cause weight gain, loss of bone density, joint problems (Bjarnason N. H. *et al.* 2008, Santen R. J. 2011) and increase the risk of thrombosis and developing cancer of the womb. Some patients may experience menopausal symptoms with Tamoxifen, such as hot flushes and vaginal dryness (Smith L. L. and White I. N. 1998, Singh M. N. *et al.* 2007).

### ***Targeted therapy***

Targeted therapy in breast cancer is, as the term suggests, the targeting of specific molecular abnormalities that are responsible for malignant progression. For many years endocrine therapy has been a successful targeted therapy with Tamoxifen

perhaps being the best example. In recent years the targeting of growth-promoting proteins such as HER2, which is over-expressed on the surface of about 15-20% of breast cancers, led to the approval for therapy with agents such as Trastuzumab (Herceptin). Herceptin, a humanized monoclonal antibody that binds the extracellular domain of the HER2 receptor, has been shown to significantly improve the clinical outcome of metastatic breast cancer patients in combination with cytotoxic therapy in the adjuvant setting (Slamon D. J. *et al.* 2001). Although Herceptin is widely used for the treatment of HER2 overexpressing breast cancer, its mechanism of action is not fully understood. The proposed mechanisms of action for Herceptin include: G1 growth arrest, inhibition of angiogenesis, induction of HER2 down regulation and activation of antibody-dependent cellular cytotoxicity (ADCC) (Valabrega G. *et al.* 2007). Other novel targeted treatments are in clinical evaluation and include anti-angiogenic compounds like Bevacizumab, Sunitinib and Vatalanib, and bi-functional drugs such as Lapatinib (small molecule inhibitor of HER2 and EGFR) (Longo R. *et al.* 2012).

### ***Immunotherapy***

With the exception of humanized monoclonal antibodies to HER2, at present no other immunotherapeutic agents have been approved for routine use in breast cancer treatment. The notion of cancer immunotherapy has a long history, with associations between tumour regression and infections dating back to the 1890s (Coley W. B. 1894). Subsequent trials included chemoimmunotherapy trials and in the 1980s, bacterial injections were combined with chemotherapy (Hortobagyi G. N. *et al.* 1983). At present, various vaccine strategies are in different stages of development (Dalgleish A. G. 2008, Kim S. H. *et al.* 2009, Wiedermann U. *et al.* 2010) and, in general, these strategies are composed of a method to deliver target antigens or epitopes to the immune system, together with an immune-stimulating adjuvant to trigger an immune response. However, as the tumour microenvironment often renders the infiltrating T cells inert (Gajewski T. F. 2006, Mellor A. L. and Munn D. H. 2008), developing strategies to overcome those immunosuppressive mechanisms and combining these with vaccines appears to be necessary. Because of the relevance to the current work, breast cancer immunotherapy will be discussed in further detail in a later section of this chapter.

In conclusion, breast cancer is a progressive disease, with a number of different subtypes that have different prognoses, but nonetheless for the majority of breast cancer patients if treatment is started quickly after an early presentation, the overall prognosis is good.

## **1.3 Glycosylation**

The term glycosylation refers to the enzymatic process that attaches glycans to proteins, lipids, or other organic molecules, and is a form of co-translational and post-translational modification. These glycans serve a variety of structural and functional roles in intracellular, membrane and secreted proteins (Varki A. *et al.* 2009). Virtually all membrane and secreted proteins are glycosylated and with the discovery of O-GlcNAc it was found that many intracellular and nuclear proteins could also carry glycans (Reason A. J. *et al.* 1992). Glycosylation increases diversity in the proteome, because almost every aspect of glycans can be modified, including the glycosidic bond, glycan composition, structure and length.

Glycosylation can be divided into a number of different types:

- 1) Glycosylation of glycolipids
- 2) Glycosylation of proteoglycans
- 3) N-linked glycans on proteins
- 4) O-linked glycans on proteins

### **1.3.1 Glycosylation of glycolipids**

Glycolipids are monosaccharide residues covalently linked to a hydrophobic moiety, such as acylglycerol, sphingoid or phenyl phosphate. In mammals, the majority of glycolipids are glycosphingolipids (GSLs) and are present in cellular membranes, typically the plasma membrane where they play a role in cell-cell recognition, cell-matrix interaction and cell surface receptor signalling (Varki A. *et al.* 2009).

Glycosphingolipid synthesis starts on the cytosolic face of the endoplasmic reticulum membrane. Ceramide is synthesized first where D-erythro-sphinganine (sphingosine-

2-amino-4-octadecene-1,3-diol- and a fatty acid) is acetylated and desaturated, prior to glucosylation by a specific ceramide glucosyltransferase to form glucosylceramide (GlcCer) or galactosylated by a specific galactosyltransferase to form galactosylceramide (GalCer) (Varki A. *et al.* 2009, Merrill A. H. 2002). GalCer extension is limited and undergoes few reactions, and  $\alpha$ -galactosylceramide and analogues have been found to be important immunomodulators (Parekh V. V. *et al.* 2013). Unlike GalCer derivatives, GlcCer can give rise to hundreds of structures and beginning with the addition of a  $\beta$ -linked galactose residue. Following galactose addition the molecules can be elongated in a stepwise fashions. A typical example is the biosynthesis of gangliosides, in which, after the action of the enzyme GalNAc transferase, several extensions can take place involving several glycosyltransferases and sialyltransferases (fig 1.3).

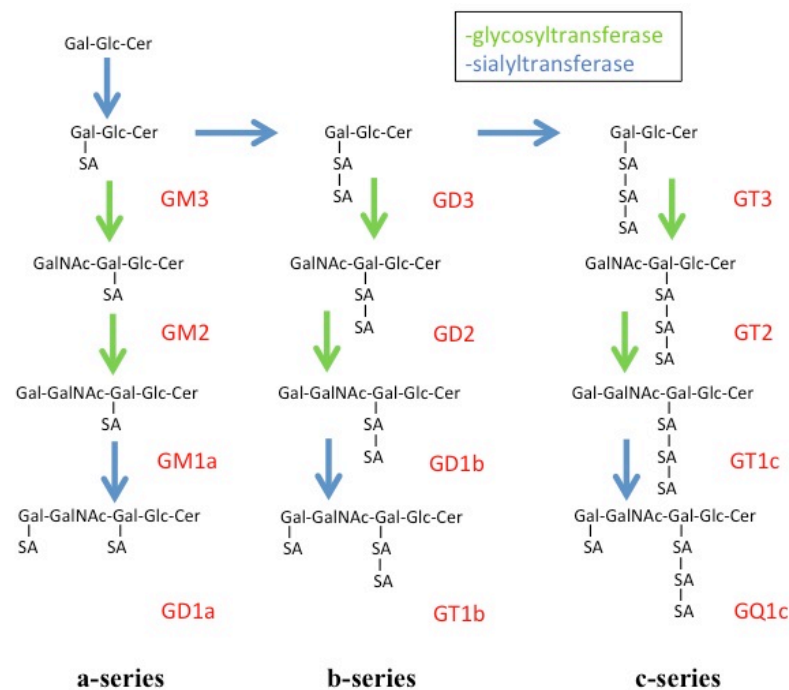


Figure 1.3 Ganglio glycosphingolipid synthesis pathway.

Alterations of these ganglioside structures have been found in different diseases, including cancers. For example it has been shown that induction of the cell surface expression of GM3 is associated with malignancy through an integrin and CD9-dependent mechanism (Mitsozuka K. *et al.* 2005).

### 1.3.2 Glycosylation of proteoglycans

Proteoglycans (PGs) are heavily glycosylated proteins, consisting of a protein moiety and one or more covalently attached, long and unbranched polysaccharides (glycosaminoglycans or GAGs). They are a major component of the extracellular matrix of multicellular organisms, where they form large complexes with hyaluronan, and fibrous matrix proteins such as collagen. PGs are involved in regulating the movement of molecules through the matrix, attract cations, bind water, and can affect the activity and stability of other proteins and signalling molecules within the matrix. Moreover, the attached GAG chains can serve as lubricants and hydrated gels enabling joints and tissues to absorb large pressure changes (Prydz K. and Dalen K. T. 2000, Simons K. and Ikonen E. 1997).

Glycosylation of the PGs occurs in the Golgi apparatus and starts by the sequential addition of xylose, galactose, galactose and glucuronic acid to a serine, where the serine residue is generally in the sequence -Ser-Gly-X-Gly- (X can be any amino acid residue). From the tetrasaccharide linker the sugar chains are extended by the addition of two alternating monosaccharides, an amino sugar and uronic acid (GlcA) (fig 1.4).

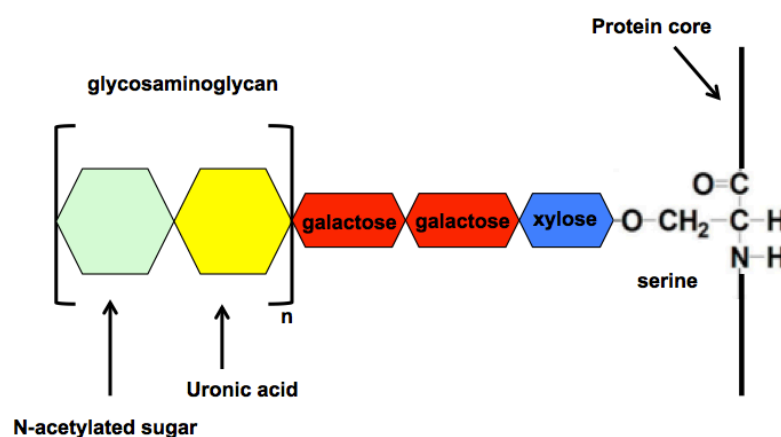


Figure 1.4 Structure of the GAG linkage to protein in proteoglycans

Proteoglycans and GAGs perform numerous vital functions within the body, for example the well-defined function of the GAG, heparin, in preventing coagulation of the blood. Because of the multitude of key roles in the normal physiology of cells



and tissue, disturbances in their metabolism can produce a variety of pathological changes, and can include symptoms such as organomegaly, heart disease, dwarfism, mental retardation and early mortality (King M. W. *et al.* 2013).

### ***1.3.3 N-linked glycosylation***

The most common type of protein glycosidic bond is N-linked, and is important for the folding of some eukaryotic proteins and for cell-cell and cell-extracellular matrix attachment. As briefly mentioned in the general introduction, N-linked glycans are covalently linked to an asparagine residue of a polypeptide with their core glycans initially added *en bloc* to proteins. Studies using peptides modified by N-glycosylation showed that the consensus sequence containing the acceptor asparagine is Asn-X-Thr/Ser, where X is any amino acid except proline (Mellquist J. L. *et al.* 1998). In general about 30% of potential N-glycosylated sites appear to be glycosylated, although this percentage varies substantially among different glycoproteins.

N-linked glycans are formed when the N-glycan lipid-linked oligosaccharide precursor structure is transferred to the nascent protein in the endoplasmic reticulum by a family of transferases. These transferases are encoded by the asparagine-linked glycosylation gene family, with this step as well as several subsequent processing reactions being conserved in all eukaryotic cells. In the cytosol the biosynthesis of the N-glycan precursor begins with the transfer of GlcNAc-P from UDP-GlcNAc by the enzyme GlcNAc-1-phosphotransferase to the dolichol phosphate (Dol-P) to generate dolichol pyrophosphate N-acetylglucosamine (Dol-P-P-GlcNAc). Following this addition, a second N-acetylglucosamine is added by the enzyme ALG14, followed by nine mannoses and three glucoses, with each step catalysed by a different transferase. The 14-sugar precursor migrates to the lumen of the ER where it is transferred to the protein by an oligosaccharyltransferase, and trimmed by endoplasmic reticulum glucosidase and mannosidase to Man5GlcNAc2. The Mgat family of GlcNAc-transferases can then add up to four branches, and its core can be modified during its passage through the Golgi (Varki A. *et al.* 2009). N-linked glycans can be further classified in three classes that share the main core sugar

sequence but differ in the chain elongation (fig 1.5): 1) high-mannose, in which only mannose residues are attached to the core; 2) complex, in which GlcNAcs are added to the mannoses by N-acetylglucosaminyltransferases; 3) hybrid, in which on one side, mannose residues are attached to Man $\alpha$ 1-6 of the core and, on the other side, GlcNAcs are attached to the Man $\alpha$ 1-3. Different portion of proteins can have different types of N-glycans, and this is thought to depend on the accessibility to saccharides-modifying proteins in the Golgi (Drickamer K. and Taylor M. E. 2005).

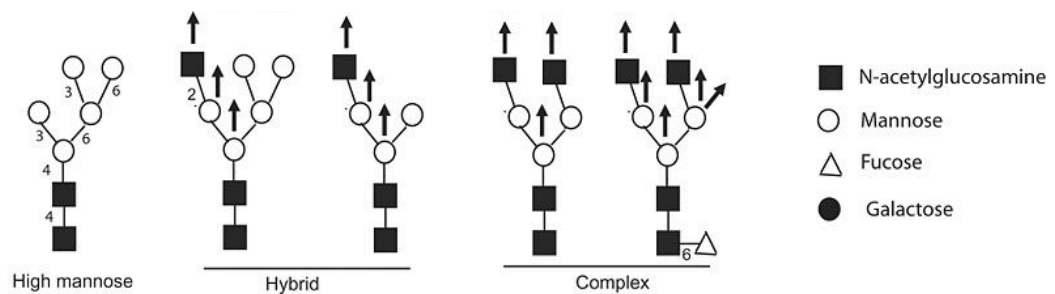


Figure 1.5 Comparative overview of major types of N-glycans in vertebrate.

Mouse models with N-glycan biosynthetic deficiency, including deficiency in the GlcNAc-1-phosphotransferase (required for building the N-glycan dolichol oligosaccharide precursor without which N-glycosylation cannot occur) resulted in embryonic lethality (Ioffe E. and Stanley P. 1994). In humans carbohydrate-deficient glycoprotein syndromes (CDGS) are relatively severe genetic diseases, caused by incomplete or failed N-glycan biosynthesis. All of these syndromes present as childhood diseases and are typically quite devastating, resulting in a failure to thrive (Jaeken J. *et al.* 1994).

### 1.3.4 O-linked glycosylation

O-linked glycosylation is the attachment of sugar molecules to an oxygen atom in an amino acid residue within a protein (Van den Steen P. *et al.* 1998). In eukaryotes, with the exception of O-GlcNAc and O-Mannosylation, O-linked glycosylation is initiated in the Golgi apparatus (Varki A. *et al.* 2009), and therefore occurs at a later stage of protein processing. Functions of O-linked glycoproteins are many and

varied, and include forming the architecture of the extracellular matrix, plus cell-cell and ligand-receptor interactions.

O-linked glycosylation can be divided into various types, which are listed in table 1.1

*Table 1.1. List of O-glycan and example of O-glycosylated protein.*

<b><i>O-Glycan Type</i></b>	<b><i>Glycoprotein Type</i></b>
O-linked GlcNAc	Nuclear and cytoplasmic
O-linked mannose	$\alpha$ -dystroglycan
O-linked fucose	EGF domains; this particular O-fucosylation is critical in the function of the receptor protein Notch
O-linked glucose	EGF domains
O-linked galactose	Collagens
Mucin-type-O-glycosylation	Plasma membrane and secreted

### ***O-linked GlcNAc***

This type consists of a single  $\beta$ -N-acetylglucosamine linked to the hydroxyl group of a serine or threonine residue, and is found on numerous cytoplasmic and nuclear proteins. At the present time, only one enzyme has been found to be involved with O-GlcNAc glycosylation, O-GlcNAc transferase (OGT), and only one enzyme is involved with its removal, D-N-acetylglucosaminidase (O-GlcNAcase). O-GlcNAc glycosylation (also called O-GlcNAcylation), is dynamic, with a very short half-life, and often competes with phosphates for the same sites. It plays an essential role in transcription with virtually every RNA polymerase II transcription factor O-GlcNAcylated (Hart G. W. *et al.* 1996, Wells L. *et al.* 2001).

An increase or decrease of GlcNAcylation due to elevated or diminished UDP-GlcNAc levels has been linked to diseases such as diabetes and cancer. In diabetes many proteins involved in PI3K/AKT signalling are modified with O-GlcNAc, and modification induces insulin resistance (Issad T. *et al.* 2010). In this way O-GlcNAc can be thought as a metabolic sensor. In cancer, tumour associated proteins like c-Myc and p53 are O-GlcNAcylated, which can inhibit phosphorylation and stabilize the protein (Chou T. Y. *et al.* 1995, Slawson C. *et al.* 2010).

### *O-linked mannose*

O-mannosylation was first identified in 1968 by Sentandreu and Northcote who isolated glycosylated proteins from the yeast cell wall (Sentandreu R. and Northcote D. H. 1968). O-linked terminal mannose is  $\alpha 1,2$ -linked to Ser or Thr and in yeast and fungi, the first mannose can be further extended to form mannotriose ( $\text{Man}\alpha 1,2 \text{Man}\alpha 1,2 \text{Man}\alpha 1\text{-Ser/Thr}$ ), with this core structure having further mannose moiety extensions depending on the yeast/fungi species (fig 1.6A) (Lommel M. and Strahl S. 2009). In mammals, O-mannosyl residues are composed of variations of the tetrasaccharide Neu5Ac-Gal-GlcNAc- $\text{Man}\alpha 1\text{-Ser/Thr}$  (fig 1.6B), can be sulphated, and are found on proteins like  $\alpha$ -dystroglycan ( $\alpha$ -DG) in nerves and muscles (Chiba A. *et al.* 1997, Sasaki T. *et al.* 1998).

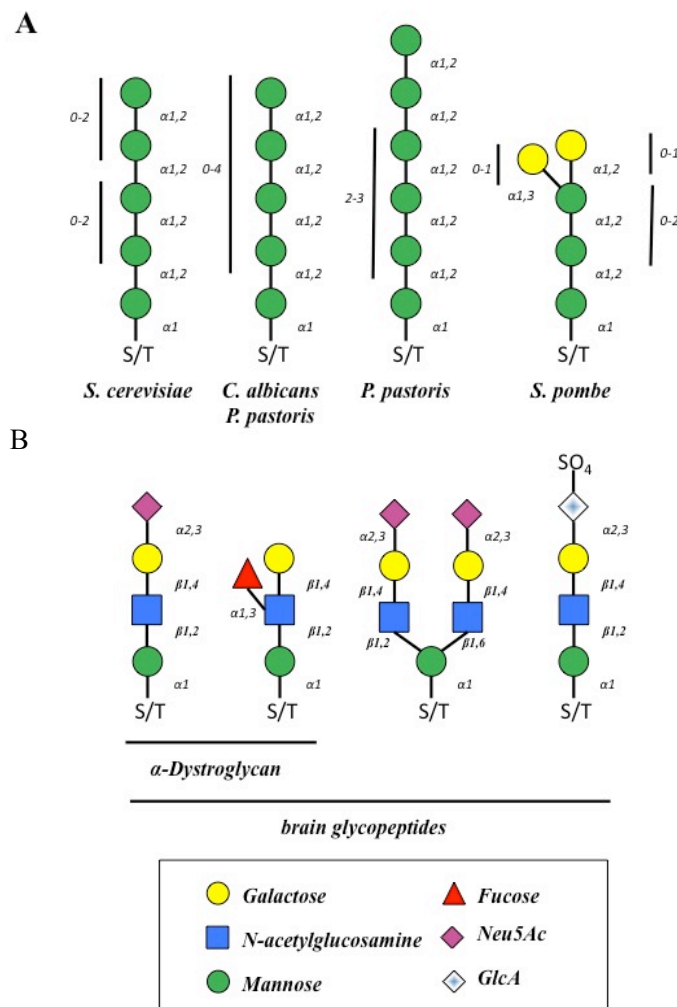


Figure 1.6 Schematic representation of O-mannosyl glycans.  
A: in yeast B: in mammals

Initiation of O-mannosylation occurs in the endoplasmic reticulum, where protein O-mannosyltransferases 1 (POMT1) and 2 (POMT2) catalyse the transfer of mannose

from Dol-P-Man to Ser/Thr residues on certain proteins. Further residue extension takes place in the Golgi apparatus. In mammals elongation of O-mannosyl glycans includes transfer of GlcNAc to mannose in a 2-OH position catalized by O-mannose N-acetylglucosaminyltransferase 1, and followed by the addition of galactose and sialic acid (Lommel M. and Strahl S. 2009).

To date, the best-studied O-mannosylated protein is  $\alpha$ -DG, which is an important component of the dystrophin-glycoprotein complex (DGC) in skeletal muscles. Glycosylation deficiency within the DGC results in muscular dystrophy, Walker Worburg syndrome (WWS), and muscle-eye-brain disease (MEB). These diseases are associated with mutation of glycosyltransferases genes such as POMT2 (Biancheri R. *et al.* 2007).

### ***O-linked Fucose***

O-fucosylation involves the addition of a fucose to a protein through the hydroxyl group of a serine or threonine residue. The consensus sequence for O-linked fucose is C-X-X-G-G-(Ser or Thr)-C, where X is any amino acid (Harris R. J. *et al.* 1993). Although only a small number of proteins have been identified with the O-linked fucose, in humans numerous secreted and cell-surface proteins contain the consensus sequence for its addition (Moloney D. J. and Haltiwanger R. S. 1999).

O-fucosylated proteins identified so far contain epidermal growth factor (EGF) like domains and thrombospondin-1 (TSP-1) repeats. These glycans have been found in proteins involved in clotting and fibrinolysis, such as urokinases, tissue plasminogen activator (t-PA), clotting factor VII, and also on Notch-1 (Varki A. *et al.* 1999). An O-fucosyltransferase (O-fut1) is responsible for the initiation of this modification, utilizing GDP-fucose, and, because this modification has been found mainly on secreted proteins, it is presumed to occur within the secretory pathway. Moreover, as GDP-fucose transporters have been found in the Golgi compartments, O-linked fucose addition to proteins is expected to occur within the Golgi (Moloney D. J. and Haltiwanger R. S. 1999). Initial reports described O-linked fucose modification as a simple monosaccharide, perhaps because fucose on other oligosaccharides in mammalian systems had always been seen as a terminal sugar, however subsequent research has shown that fucose can be further extended. For example, human clotting factor IX contains the tetrasaccharide fucose structure Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-

4GlcNAc $\beta$ 1–3Fuc $\alpha$ -, and on proteins derived from Chinese hamster ovary (CHO) cells, fucose was found to be elongated with a  $\beta$ 1,3 linked glucose residue (Harris R. J. *et al.* 1993, Moloney D. J. *et al.* 1997).

O-fucosylation is particularly important for the protein receptor Notch, which plays essential roles in many phases of development. Mutation of Notch or its downstream signalling pathway has been implicated in a multitude of diseases, including Leukemia, Multiple Sclerosis, and Breast Cancer (Rana N. A. and Haltiwanger R. S. 2011, Rampal R. *et al.* 2007). O-fucose addition by POFUT1 is absolutely necessary for Notch function, and POFUT1 mutations results in loss of Notch signalling (Yao D. *et al.* 2011, Okajima T. and Irvine K. D. 2002). GlcNAc elongation at the O-fucose by the  $\beta$ 1-3N-acetylglucosaminyl transferase, Fringe, has been shown to modulate Notch activity in a number of tissues (Moloney D. J. *et al.* 2000, Bruckner K. *et al.* 2000), and alter Notch-ligand binding (Stanley P. and Okajima T. 2010).

### ***O-linked glucose***

Similarly to O-fucosylation, O-glucosylation has been found on TSP-1 repeats and EGF-like domains, although these two types of glycosylation have been found to occur together only on EGF-like domains. Proteins such as Notch, factor VII, factor IX,  $\delta$ -like protein 1, and protein Z contain O-linked glucose on their EGF-like repeats, and in  $\delta$ -like protein 1 and factor VII the presence on a single EGF-like domain of both O-fucose and O-glucose has been shown (Bjoern S. *et al.* 1991, Krogh T. N. *et al.* 1997).

Proteins are O-glucosylated at a specific serine of the EGF-like repeats, and the O-glucosylated consensus motif is C<sup>1</sup>-X-S-X-P-C<sup>2</sup>, where C<sup>1</sup> and C<sup>2</sup> are the first and second cysteine of the EGF domain. Furthermore, the importance of O-glucosylation was demonstrated in recent years after the discovery of the O-glucosyltransferase, Rumi, as in its absence, Notch signalling is abolished (Acar M. *et al.* 2008). All the O-glucosylated sites, not just a single one, contribute to a robust Notch signalling (Leonardi J. *et al.* 2011).

### ***O-linked galactose***

O-galactosylation is defined by the building of an O-linked galactose on hydroxylysine residue, with the O-galactosylated consensus motif being Gly-Xaa-Hyl-Gly (Michaelsson E. *et al.* 1994). Currently, only a few proteins have been found to be O-galactosylated, including collagen and surfactant (Varki A. *et al.* 2009). O-galactosylation occurs in the endoplasmic reticulum, and is catalysed by two galactosyltransferases GLT25D1 and GLT25D2. The GLT25D1 gene is constitutively expressed in all human tissue, while GLT25D2 gene is found to be expressed at a low level in the human nervous system (Schegg B. *et al.* 2009).

### ***Mucin-type O-linked glycoproteins***

Mucins are the most common group of O-glycosylated proteins in humans (Brockhausen I. 1999, Wopereis S. *et al.* 2006), and their glycosylation is termed mucin-type-O-glycosylation. The synthesis of mucin-type-O-glycosylation is initiated by a family of enzymes called peptidyl N-acetylgalactosaminyltransferases (ppGalNAcTs) which transfer an N-acetylgalactosamine (GalNAc) moiety from UDP-GalNAc to a serine or threonine residue. The first ppGalNAc gene to be cloned and characterised from bovine tissue was a type II transmembrane protein, and the amino acid sequence showed little homology with other glycosyltransferases cloned at the time (Hagen F. K. *et al.* 1993). When the ppGalNAc gene was knocked out in a mouse model, the mouse showed no phenotypic differences, suggesting complexity in O-glycan biosynthesis that must involve multiple enzymes with overlapping specificities (Hennet T. *et al.* 1995). Since then, many more ppGalNAcT genes have been cloned with distinct but overlapping specificities (Sorensen T *et al.* 1995, Clausen H. and Bennett E. P. 1996, Wandall H. H. *et al.* 1997). Comparison of amino acid sequences of ppGalNAcTs from mammalian homologues were found to be between 97 to 100% (Hagen F. K. *et al.* 1995), and analysis of enzyme kinetics by capillary electrophoresis and mass spectrometry showed that various ppGalNAcTs react at different rates with individual sites in the tandem repeats of mucins (Wandall H. H. *et al.* 1997). Following GalNAc addition, this sugar is used as a substrate for the addition of different glycans to form various core structures which are listed in table 1.2, and the glycosyltransferases involved in the early steps of mucin-O-linked glycosylation are listed in table 1.3.

Table 1.2 Core O-glycan structures

<b>Core</b>	<b>Linkage</b>
1	Gal $\beta$ 1,3GalNAc
2	(GlcNAc $\beta$ 1,6)Gal $\beta$ 1,3GalNAc
3	GlcNAc $\beta$ 1,3GalNAc
4	(GlcNAc $\beta$ 1,6)GlcNAc $\beta$ 1,3GalNAc
5	GalNAc $\beta$ 1,3GalNAc
6	GlcNAc $\beta$ 1,6GalNAc
7	GalNAc $\alpha$ 1,6GalNAc
8	Gal $\alpha$ 1,6GalNAc

Table 1.3 Key glycosyltransferases involved in the early steps of mucin O-linked glycosylation

<b>Name</b>	<b>Abbreviated name</b>	<b>Reaction</b>	<b>Comments</b>
UDP-N-acetyl- $\alpha$ -D-galactosamine: polypeptide N-acetylgalactosamyltransferases	GalNAc-transferase or GalNAcTs	GalNAc to serine or threonine in alpha linkage to form <b>Tn</b>	Large family of enzymes, designated GalNAcT1, T2, T3 etc.
CMP-Neu5Ac:GalNAc-R $\alpha$ 2,6-sialyltransferase-I	ST6GalNAc-I	Sialic acid to GalNAc-Ser/Thr in an $\alpha$ 2,6 linkage to form <b>STn</b>	At present this is the only enzyme shown to synthesize STn
Core 1 $\beta$ 3-Gal-transferase	$\beta$ 1,3GalT	Gal to GalNAc in $\beta$ 1,3 linkage to form <b>core1 (T)</b> .	Require expression of a molecular chaperone designated Cosmc
CMP-NeuAc: Gal $\beta$ 1,3GalNAc $\alpha$ 2,3-sialyltransferase-I	ST3Gal-I	Sialic acid to Gal of core 1 in $\alpha$ 2,3 linkage to form <b>sialyl core 1 (ST)</b>	Compete with C2GnT1 for the same substrate in the mammary glands. ST3Gal-II has the same specificity but is not expressed by the mammary gland.
CMP-Neu5Ac: GalNAc-R $\alpha$ 2,6-sialyltransferase-II	ST6GalNAc-II	Sialic acid in $\alpha$ 2,6 linkage to the GalNAc of core 1 or sialyl core 1 to form <b>disialyl core 1 (disialyl T)</b>	Mouse ST6GalNAc-II can also make STn. This has not been demonstrated for the human enzyme.
Core 2 $\beta$ 1,6-N-acetylglucosamyl transferase 1	C2GnT1	GlcNAc to the GalNAc of core 1 in $\beta$ 1,6 linkage to form <b>core 2</b> .	The C2GnT1 expressed by the mammary gland compete with ST3Gal-I for the same substrate.
Core 2/4 $\beta$ 1,6-N-acetylglucosamyl transferase	C2GnT2 or C2/4GnT	GlcNAc to GalNAc of core 1 or core 3 in $\beta$ 1,6 linkage to form <b>core 2or core 4</b> respectively.	Does not appear to be expressed by the mammary gland.
Core 2 $\beta$ 1,6-N-acetylglucosamyl transferase 3	C2GnT3	GlcNAc to GalNAc of core 1 in $\beta$ 1,6 linkage to form <b>core 2</b> .	Only occasionally expressed by the mammary gland
Core 3 $\beta$ 1,6-N-acetylglucosamyl transferase	C3 $\beta$ 3GnT	GlcNAc to GalNAc in $\beta$ 1,3 linkage to form <b>core 3</b> .	Not shown to be expressed by the mammary gland.



Further elongation of core structures by specific transferases is highly regulated, for example the core 2 structure is usually extended with a further Gal residue on C4 of the GlcNAc. This is followed by a variable number of lactosamine chains that can be terminally fucosylated by  $\alpha$ 3-Fuc-transferase ( $\alpha$ 3-FucT) followed by sialylation of an  $\alpha$ 3-sialic acid to Gal by an  $\alpha$ 3-sialyltransferase (ST3Gal), to form the Le<sup>x</sup> or sLe<sup>x</sup> structures (fig 1.7). Extension of core 2 structures can also occur from the galactose, which can be terminally fucosylated by  $\alpha$ 4-Fuc-Transferase ( $\alpha$ 4-FucT) followed by sialylation of an  $\alpha$ 3-sialic acid to Gal by an  $\alpha$ 3-sialyltransferase (ST3Gal) to form the structures Le<sup>a</sup> or SLe<sup>a</sup>. Branching of the polylactosamine backbone can occur.

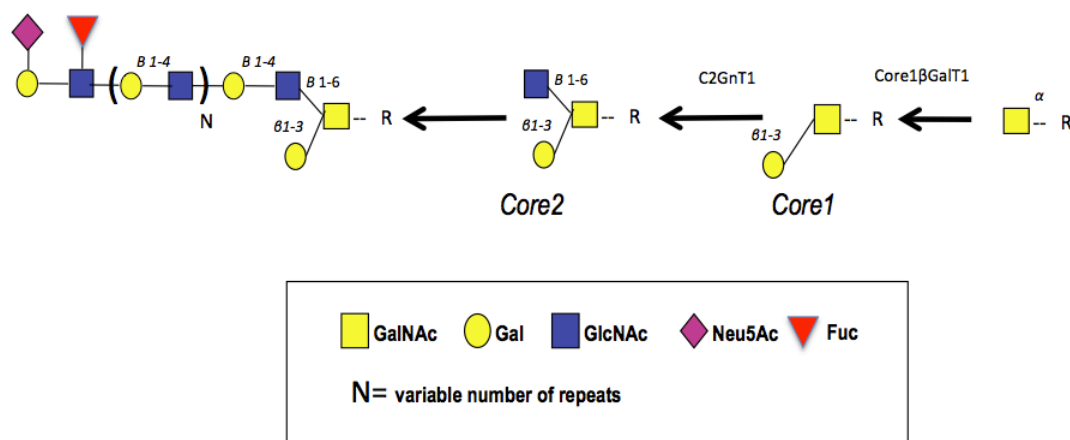


Figure 1.7 Example of O-glycosylation extension (SLe<sup>x</sup>)

In cancer many changes in O-linked glycosylation occurs, and those changes will be discussed further later in this chapter.

## 1.4 Mucins

Mucins are proteins with a high content of O-linked carbohydrates where GalNAc is linked to serine and threonine. The carbohydrate content typically makes up between 50 to 90% of the mucin's total molecular weight. There are currently 20 members in the mucin family (table 1.4). Physiologically, mucins are important in protection from pathogens, lubrication, prevention of water loss and cell-cell interactions, which is reflected by their expression patterns (table 1.4). Broadly, mucins can be split into two groups: secreted mucins (gel-forming) and membrane bound (non gel-forming). A feature common to all the mucins is the presence of tandemly repeated

amino acid sequences, which contain a high level of serine and threonine residues potential sites of O-glycosylation. These tandem repeats are used as a scaffold for large numbers of O-linked carbohydrate chains. In many mucins the number of tandem repeats is variable so this domain is known as the VNTR (variable number of tandem repeats).

**Table 1.4** *Currently identified mucins.*

The chromosomal localisation, tissue expression, number of amino acids in the tandem repeat and the nature of the mucin (secretory (s) or membrane bound (m)) are shown.

<b>Mucin Name</b>	<b>Secreted (s) Membrane bound (m)</b>	<b>Gene Location</b>	<b>No. aas in tandem repeat</b>	<b>Normal tissue expression</b>	<b>Reference</b>
Mucin 1	m,s	1q21-q24	20	Most simple epithelial cells, some leukocytes	(Gendler S. J. <i>et al.</i> 1987)
Mucin 2	S	11p15	23	GI tract, respiratory tract	(Gum J. R. <i>et al.</i> 1994)
Mucin 3A	m,s	7q22	17	GI tract	(Van Klinken B. J. <i>et al.</i> 1997)
Mucin 3B	m,s	7q22	17	GI tract	(Kyo K. <i>et al.</i> 2001)
Mucin 4	m,s	3q29	16	Trachea, colon, bronchus, breast	(Nollet S. <i>et al.</i> 1998)
Mucin 5AC	S	11p15	8	Respiratory, rectum, eye, stomach	(Guyonnet Duperat V. <i>et al.</i> 1995)
Mucin 5B	S	11p15	87	Respiratory, Stomach	(Meezaman D. <i>et al.</i> 1994)
Mucin 6	S	11p15.5-15.4	169	Bile duct, stomach, colon, endocervix	(Toribara N. W. <i>et al.</i> 1993)
Mucin 7	S	4q13-q21	22	Salivary gland	(Bobek L. A. <i>et al.</i> 1993)
Mucin 8	S	12q24.3	?	Trachea, nasopharynx	(Shankar V. <i>et al.</i> 1994)
Mucin 9	M	1p13	15	Trachea, oviduct	(Lapensee L. <i>et al.</i> 1997)
Mucin 11	M	7q22	28	GI tract	(Williams S. J. <i>et al.</i> 1999)
Mucin 12	m,s	7q22	28	GI tract, uro-genital tract	(Williams S. J. <i>et al.</i> 1999)
Mucin 13	M	3q13.3	?	GI tract, urogenital tract	(Williams S. J. <i>et al.</i> 2001)
Mucin 15	M	11p14.3	?	GI tract	(Pallesen L. T. <i>et al.</i> 2002)
Mucin 16	M	19p13.3	156	Eyes, Ovaries	(Davies J. R. <i>et al.</i> 2007)
Mucin 17	M	7q22	?	Ovaries	(Gum J. R. <i>et al.</i> 2002)
Mucin 19	S	12q12	?	Trachea, salivary gland	(Chen Y. <i>et al.</i> 2004)
Mucin 20	M	3q29	?	Kidneys, placenta, lungs, prostate, liver	(Higuchi T. <i>et al.</i> 2004)
Mucin 21	M	11p15	15	Bronchi, bronchioli, large intestine, thymus, testis.	(Itoh Y. <i>et al.</i> 2008)

### ***1.4.1 Secreted mucins***

The MUC2, MUC5AC, MUC5B and MUC6 secreted mucins are thought to share a common ancestor and are encoded by a cluster of genes at the chromosomal locus 11p15 (Desseyn J. L. *et al.* 2000). Many of these secreted mucins contain cysteine rich domains that are responsible for dimerization in the endoplasmic reticulum and oligomerization in the Golgi, which results in the formation of a viscous gel. This provides lubrication, prevents dehydration and inhibits the access of bacteria to the cell surface.

The functions of secreted mucins are dependent on their ability to form viscous solutions or gels. The highly glycosylated domains are devoid of secondary structures and secreted mucins are long extended structures that are much less flexible than unglycosylated random coils. The oligosaccharides contribute to this stiffness in two ways, by limiting the rotation around peptide bonds and by charge repulsion among the neighbouring negatively charged oligosaccharide groups (Jentoft N. 1990). Such long, extended molecules have a much greater solution volume than native or denatured proteins with little or no carbohydrate, resulting in highly viscous aqueous mucin solutions.

MUC2 has been found to be the main mucin of the intestinal mucus, organized in a sophisticated two-layer system. The outer layer has been found to provide a good symbiotic environment in which bacteria can thrive, while the inner mucus is well-structured and virtually impermeable to bacteria (Johansson M. E. and Hansson G. C. 2011). Mucins protect against infection by microorganisms that bind to carbohydrates, and mucin genes appear to be up-regulated by substances derived from bacteria, e.g. lipopolysaccharides (Lieleg O. *et al.* 2012, Radziejewska I. 2012, Dohrman A. *et al.* 1998).

### ***1.4.2 Membrane bound mucins***

Membrane bound mucins, or transmembrane mucins, such as MUC1, MUC3A and B, MUC4 and MUC16 are membrane bound due to the presence of a hydrophobic

membrane-spanning domain that favours retention in the plasma membrane. The extracellular domains of membrane bound mucins protrude far into the extracellular space, for example MUC1 extends between 200 to 500nm from the cell surface and so far beyond the 30nm glycocalyx. It is the relatively rigid structure, which is the result of high number of proline residues and clustered glycans, which allows the membrane mucins to extend far above the cell surface (Godula K. *et al.* 2009). Generally all mucins have a protective role, but membrane bound mucins additionally interact with many different proteins inside and/or outside the cell, and are involved in modulation of cell-cell and protein-protein interactions and in the regulation of various downstream signalling events.

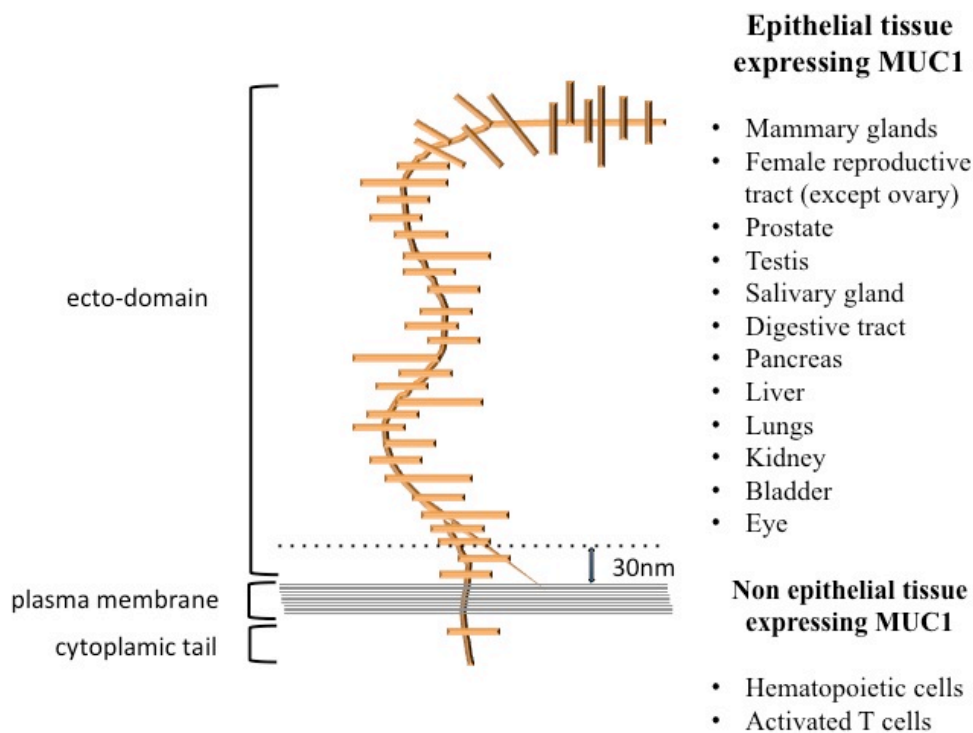
### **1.4.3 MUC1**

Human mucin-1 (MUC1) was first identified as a major component of milk fat globule membranes by Shimizu and Yamauchi in 1982, and during the early years of its discovery was also known by other names including epithelial membrane antigen (EMA) (Ormerod M. J. *et al.* 1983), polymorphic urinary mucin (PUM) (Swallow D. M. *et al.* 1987) and episialin (Ligtenberg M. J. *et al.* 1990). Several antibodies to MUC1 were developed using epithelial cells or membrane extracts derived from them. These antibodies were used in expression cloning to clone the partial cDNA of MUC1 (Gendler S. J. *et al.* 1987) followed by cloning of the full-length sequence (Gendler S. J. *et al.* 1991) and this allowed the prediction of its amino acid sequence.

The MUC1 gene is localized on chromosome 1q21 (Swallow D. M. *et al.* 1987), and is translated as a single protein before being cleaved in the ER into two subunits (N-terminal and C-terminal subunit), that are held together on the cell surface in a heterodynamic complex by strong non-covalent sodium dodecyl sulphate (SDS)-labile bonds (Hattrup C. L. and Gendler S. J. 2008, Levitin F. *et al.* 2005). Like many mucins MUC1 has a variable number of tandem repeats, which is allele dependent, varying from 20 to 125 (Gendler S. J. *et al.* 1987; Burchell J. M. *et al.* 2001). Like all the mucins, MUC1 has a high percentage of carbohydrates (more than 50% by mass) predominantly O-linked to serine or threonine residues (Taylor-Papadimitriou J. *et al.* 2002). The dense glycosylation is crucial for its protective functional role, giving

a bottlebrush-like structure, allowing it to extend 200-500nm from the cell surface (fig 1.8), far more than the glycocalyx (30nm) and surface receptors (Singh P. K. and Hollingsworth M. A. 2006) and contributing to its binding, water-holding, protease insensitivity and pathogen binding properties (McAuley J. L. *et al.* 2007, Linden S. K. *et al.* 2009, Patton S. *et al.* 1995).

In response to environmental signals, the large MUC1 N-terminal subunit can be shed from the cell surface (Abe M. and Oshima R. G. 1990), and the MUC1 C-terminal subunit is associated with intracellular signalling. These intracellular activities are found to play important roles in the physiology of both malignant and non-malignant cells (Carson D. D. 2008), including functioning as a substrate for epidermal growth factor receptor (EGFR) mediated signalling (Li Y. *et al.* 2001), and other tyrosine kinase receptors.



*Figure 1.8 Schematic diagram of MUC1 and sites of expression*  
(Figure adapted from Brayman, M. *et al.* 2004).

### ***MUC1 Protein structure***

MUC1 is a type I membrane bound glycoprotein. At the C-terminal its cytoplasmic tail is composed of 72 amino acids (aa) and the transmembrane domain has 31aa. The cytoplasmic domain shows a high degree of homology between mouse and

human indicating conserved functions. The MUC1 precursor is proteolytically cleaved in the ER, separating the cytoplasmic domain, the transmembrane domain and the first 65aa of the extracellular domain from the rest of the precursor (Ligtenberg M. J. *et al.* 1992, Levitin F. *et al.* 2005, Macao B. *et al.* 2006). The two subunits of MUC1 then form a stable non-covalent complex, which is expressed on the apical surface of normal simple epithelial cells. The major region of the extracellular domain is made up of VNTRs of 20-conserved amino acids, rich in the serine and threonine residues that are sites of potential O-glycosylation (Tandem repeat amino acids sequence HGVTSAPDTRPAPGSTAPPA). Flanking the conserved tandem repeats, there are degenerate tandem repeats differing only by few amino acids (Gendler S. J. *et al.* 1990).

The polymorphic nature of MUC1 can be seen at the RNA, DNA and protein level, and has been shown to be due to the variable number of VNTRs, with the number differing depending on the allele (Swallow D. M. *et al.* 1987, Gendler S. J. *et al.* 1990). In heterozygote individuals, two bands can be observed at the DNA level (Southern blots), RNA level (Northern blot) and protein level on SDS gels, due to the co-dominant expression of alleles (Karlsson S. *et al.* 1983). The mass of the core protein without glycosylation has been found to be between 120-225 kDa (Gendler S. J. *et al.* 1990). In breast cancer, with the attachment of carbohydrates the size of MUC1 can range between 250-500 kDa, and much more in normal epithelial expression due to the extended glycosylation.

Both N-and O-linked glycosylation occurs on MUC1, with O-glycans being the most abundant. MUC1 glycosylation starts during translation, with the addition of N-glycans in the endoplasmic reticulum which are modified in the Golgi, while the O-linked glycosylation, as described in the O-linked glycosylation section of this introduction, occurs in the Golgi apparatus (Rose M. C. and Voynow J. A. 2006). In polarized epithelial cells MUC1 is translocated to the luminal surface, and the first three residues (CQC) of the cytoplasmic domain are involved in retention at the plasma membrane (Pemberton L. F. *et al.* 1996). MUC1 ectodomain glycosylation is found to affect MUC1 cell surface expression by slowing down delivery through the biosynthetic pathway and by increasing its internalization (Altschuler Y. *et al.* 2000). MUC1 tandemly repeated core glycosylation is found to be an apical target for

signals delivered upon binding to lectins such as the tomato lectin (*Lycopersicon esculentum*; *LEA*) and wheat germ agglutinin (*Triticum vulgare*; *WGA*) (Kinlough C. L. *et al.* 2011).

In addition to the high levels of serine and threonine residues, MUC1 is also rich in proline (about 25% of the total aa), which contributes to its extended structure and enables the formation of  $\beta$ -sheets. Proline inhibits the formation of  $\alpha$ -helix structures and therefore promotes an extended conformation allowing the access of glycosyltransferases. In addition, the tandem repeat domain of MUC1 does not contain any hydrophobic residues such as Trp, Ile and Phe, further promoting a rigid and extended protein structure.

### ***Expression***

MUC1 is mainly expressed on the apical surface of the epithelial cells lining glands or ducts in normal tissues, including the mammary gland (Patton S. *et al.* 1995). A very low level of expression has been observed in resting (Chang J. F. *et al.* 2000) and activated T cells (Agrawal B. *et al.* 1998, Correa I. *et al.* 2003). However, contrary to the findings by Chang and colleagues, the study by Correa and colleagues showed that resting T cells were negative for MUC1 expression at both the protein and mRNA level. MUC1 expression was shown to be induced on activated T cells, but found to be 50 times lower than that expressed by autologous breast cancer. With regard to the present work, MUC1 expression on T cells is of substantial importance, when considering MUC1 based immunogen for immunotherapy, because it could theoretically lead to immune tolerance. This is based on the assumption that if T cells express MUC1, those cells may deplete each other if primed to MUC1. Recently, MUC1 has been associated with the development of different regions of the terminal respiratory tract and on a population of stem cells (Engelmann K. *et al.* 2008, Hikita S. T. *et al.* 2008), suggesting a developmental role. However, in the mammary glands, MUC1 is a marker of differentiation.

MUC1 is highly expressed by the majority of carcinomas, including breast, pancreas, ovary, lung and colon (Girling A. *et al.* 1989), and is also present in some non-epithelial malignancies such as about 30% of sarcomas and brain tumours and 20%

of lymphomas (Zotter S. *et al.* 1988). In a human breast cancer cell line, the levels of MUC1 protein correlate with mRNA levels (Abe M. and Kufe D. 1990), indicating that the overexpression of MUC1 observed in cancer is controlled at the level of transcription. In adenocarcinomas, when the cancer cell loses its polarity, MUC1's apical distribution is lost and its expression is found all over the cell and in the cytoplasm (Rahn J. J. *et al.* 2001). In addition to its high expression in the majority of carcinomas, MUC1 has also been detected on some T cell lymphomas and myelomas associated with the development of CD8 memory T cells specific for MUC1 (Takahashi T. *et al.* 1994, Choi C. *et al.* 2005), and a small percentage of plasma cells (Hilkens J. *et al.* 1995).

#### **1.4.4 Signalling function of MUC1 and other membrane mucins**

In normal polarized epithelial cells transmembrane mucins are found on the luminal surface and are therefore spatially separated from many of the growth factor receptors. However, in malignancy when polarity is lost, mucins can come into contact with these receptors. Some membrane mucins such as MUC1 and MUC4, have been found to interact with other receptor families, such as the receptor tyrosine kinases ErbB1 (EGFR) and ErbB2, to enhance cancer cell proliferation. As diagrammatically described in figure 1.9 (taken from Bafna S. *et al.* 2010), MUC1 interacts through its cytoplasmic tail (CT) with EGFR increasing cell proliferation via activation of extracellular signal-regulated kinases (Erks) (Pochampalli M. R. *et al.* 2007). MUC1 has also been found to be involved in the nuclear localisation and function of EGFR (Bitler B. G. *et al.* 2010). The cytoplasmic tail of MUC1 (MUC1-CT) can also interact with  $\beta$ -catenin, which inhibits  $\beta$ -catenin interacting with GSK3 $\beta$ -mediated degradation (Ren J. *et al.* 2006). The MUC1-CT/ $\beta$ -catenin complex has also been found to influence transcription by binding to chromatin within the nucleus where the complex influences cell proliferation (Ren J. *et al.* 2006).

Figure 1.9 (Bafna S. *et al.* 2010) describes diagrammatically how MUC4 has been shown to contribute to increased cell proliferation through ErbB2 and subsequent activation of Erk and Akt signalling pathways (Chaturvedi P. *et al.* 2008, Ramsauer



V. P. *et al.* 2006). Moreover, MUC4 has been proposed as an activation ligand for ErbB2, due to the fact that its expression in some cell lines elevates ErbB2 tyrosine phosphorylation (Albrecht H. and Carraway K. L. 2011).

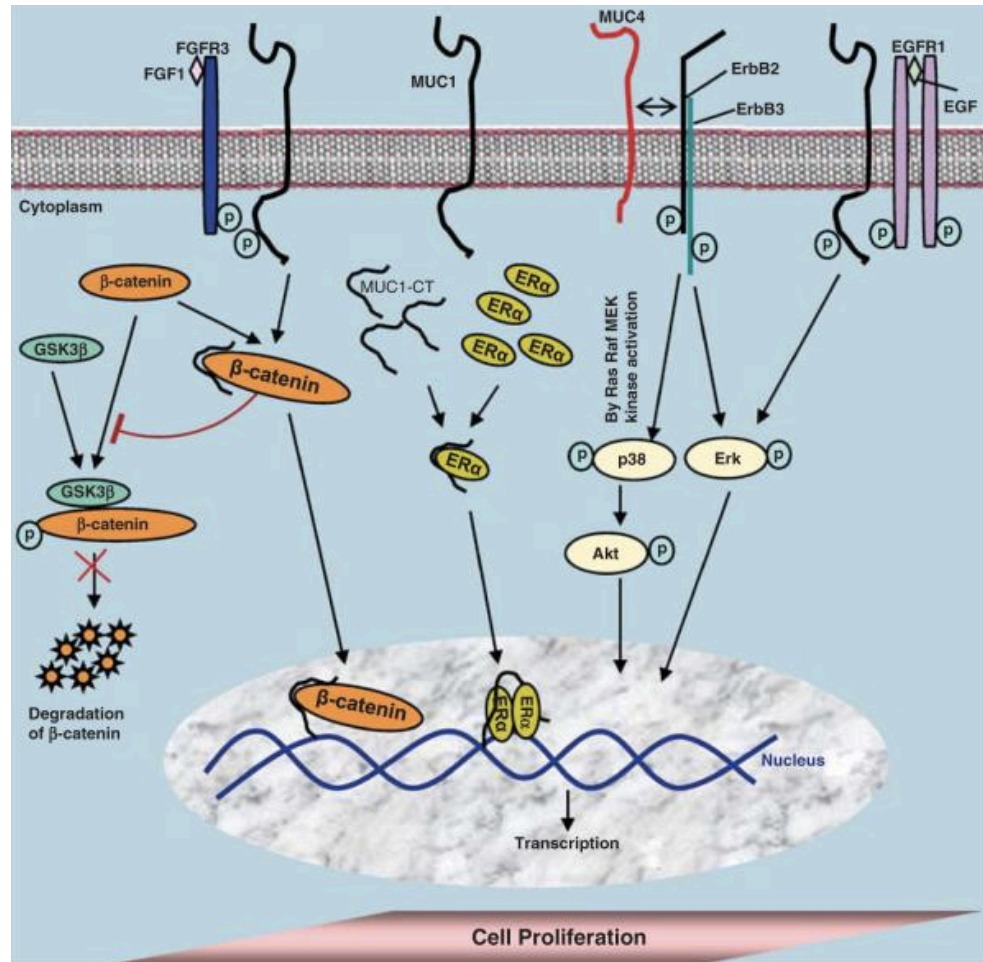


Figure 1.9 Examples of transmembrane mucin-mediated cellular functions (1)

Proposed MUC1 and MUC4 mechanisms for enhanced cell proliferation (Figure taken from Bafna S. *et al.* 2010).

In addition to increasing cell proliferation, MUC1 and MUC4 have also been shown to repress apoptosis by distinct mechanisms. As shown in figure 1.10, MUC1-CT suppresses the induction of apoptosis by activating p53 through direct binding, which in turn selectively decreases transcription of apoptotic genes as a survival response to stress and thereby decreases cell death (Wei X. *et al.* 2005). Also, MUC1 activates the FOXA3a survival-related transcription factor in response to oxidative stress by inhibiting its phosphorylation (Yin L. *et al.* 2004). Direct interaction of the MUC1 cytoplasmic tail with Inhibitor Kappa B (IκB) kinase complex can cause degradation of this complex. Thus nuclear factor-kB (NF-kB) is released from IκB and is free to

act as a transcription factor blocking apoptosis and inducing transformation (Ahmad R. *et al.* 2007). In addition, MUC1 has been shown to block nuclear targeting of c-Abl, which blocks apoptosis (Raina D. *et al.* 2006). Furthermore, MUC1-CT is also thought to attenuate the mitochondrial apoptotic pathway through binding of HSP90 (Ren J. *et al.* 2006), and by interacting with the death domain of FADD (Agata N. *et al.* 2008). MUC4 has been proposed to repress apoptosis by up-regulation of Kip (Chaturvedi P. *et al.* 2007).

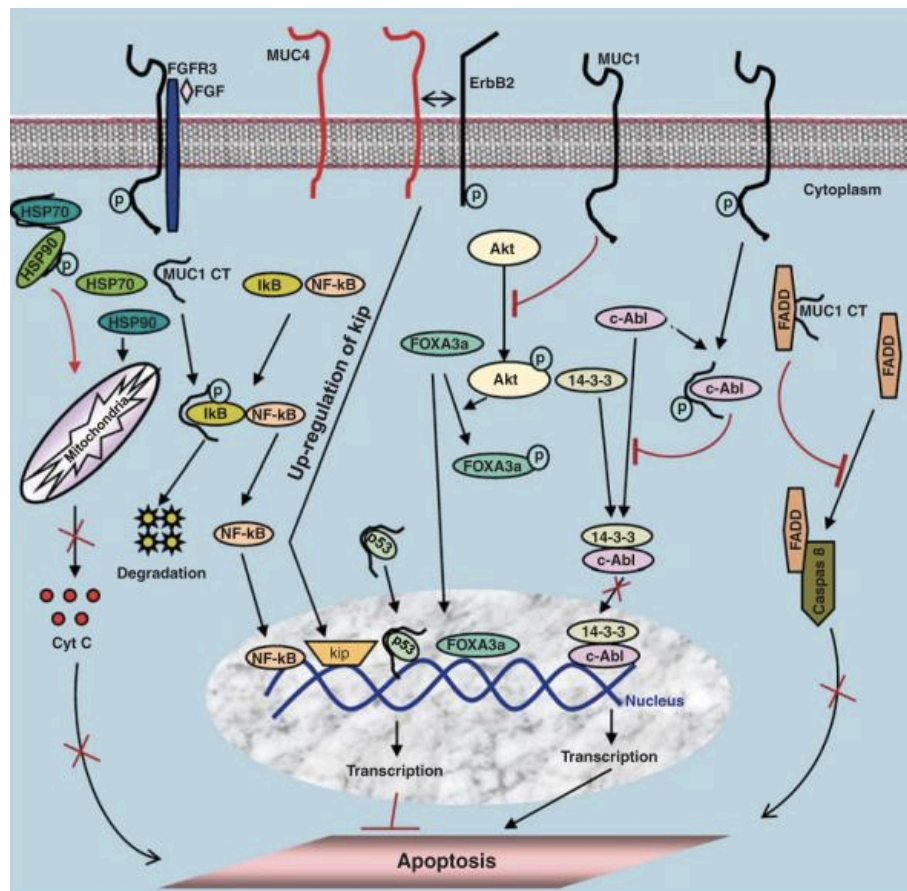


Figure 1.10 Examples of transmembrane mucin-mediated cellular functions (2)

Proposed MUC1 and MUC4 mechanisms for the repression/activation of apoptosis (Figure taken from Bafna S. *et al.* 2010).

Additional proposed regulation of various downstream signalling events, investigated in murine models, includes the direct binding of Muc1 to effector proteins like c-Src, tyrosine kinases and  $\beta$ -catenin. Indeed in a spontaneous mammary tumour mouse model (PyMT) Muc1 was found to interact with c-Src. Importantly c-Src is essential for the induction of mammary tumours in this model and in Muc1 null mice the development of PyMT induced tumours is significantly

delayed. These interactions have been shown to induce the activation of a variety of effector proteins including phosphatidylinositol 3-kinase, Shc, and STAT3 (fig 1.11), and induce the transcription of a variety of genes involved in mitogenesis, differentiation, apoptosis and quiescence (Senapati S. *et al.* 2010, Al Masri M. and Gendler S. J. 2005).

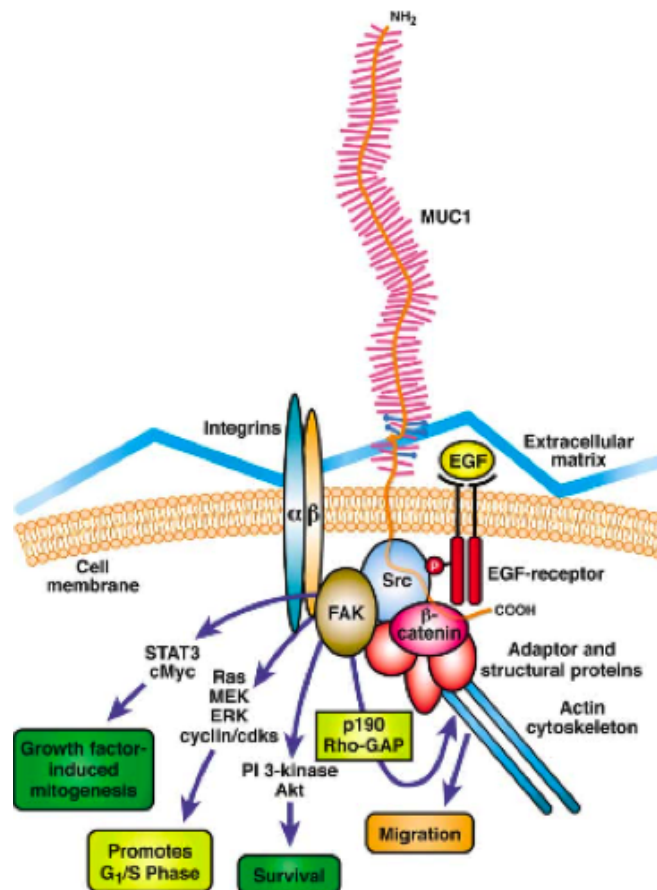


Figure 1.11 Schematic representation of Muc1 cytoplasmic tail interactions (Al Masri M. and Gendler S. J. 2005)

## 1.5 Aberrant glycosylation in cancer

Change in cellular glycosylation is a universal feature of malignant transformation and tumour progression, and is considered as an essential mechanism in defining stage, direction, and fate of tumour progression (Ono M. and Hakomori S. 2004). Many studies have shown a clear correlation between aberrant glycosylation status and invasive/metastatic potential of cancers (Hakomori S. 1996). Moreover, the changes that occur in cancer can take a variety of forms. Cancer cells can lose or

increase expression of certain structures, can express incomplete or truncated structures can have an accumulation of precursor structures and the appearance of novel structures can be observed. Every type of glycosylation (see section 1.3) can be altered in tumorigenesis, and perhaps some of the most common and highly investigated changes include: increased  $\beta$ 1,6GlcNAc branching in N-linked structures, resulting from enhanced or induced expression of GlcNAc transferase-V (Dennis J. W. 1988); alteration of ganglioside glycosylation resulting in an increase in GD3 expression in brain tumours and melanoma (Walker P. R. *et al.* 2002, Ohkawa Y. *et al.* 2010); and with particular importance to the work described in this thesis, changes in O-linked glycans attached to mucins.

Early events in the evolution of neoplasia involve alterations in oncogenes and other genes, and subsequent tumour growth, invasion, and metastasis can perhaps be regarded as ‘survival of the fittest’ on a cellular level. It is likely that changes in tumour cell glycosylation can have the greatest functional consequence during subsequent tumour growth, rather than during early events, although this appears not always to be the case. Indeed, evidence of this comes from the significant correlations between certain types of altered glycosylation and the prognosis of tumour-bearing animals or patients. Although, at present time the mechanism by which these glycosylation changes alter tumour behaviour is currently not fully understood, data are emerging and will be further introduced in this chapter and chapter 5 of this thesis describes how overexpression of the glycosyltransferase ST3Gal-I in the mammary gland leads to increased mammary tumours.

### ***1.5.1 Aberrant glycosylation of MUC1 in breast cancer***

In normal breast epithelial cells the O-linked glycans attached to MUC1 are core 2 based (see 1.3.4 O-linked glycosylation). However, MUC1 expressed by breast carcinomas has an increased proportion of shorter, non-branched O-glycans with a higher sialic acid content, often being core 1 based (Brockhausen I. *et al.* 1995, Hanisch F. G. *et al.* 1996, Lloyd K. O. *et al.* 1996, Muller S. and Hanisch F. G. 2002). In breast carcinomas the glycan chain on MUC1 can stop after the addition of the first glycan to give Tn (Sorensen A. L. *et al.* 2006), or the formation of the core 1

structure to give T (sometimes called TF). However, as the majority of MUC1 has a higher sialic acid content, glycan chains can be terminated through the action of the ST6GalNAc-I or ST3Gal-I sialyltransferases, which add sialic acid to the GalNAc or to the core 1 structure respectively. ST6GalNAc I, II and IV can further sialylate on a C6 of the GalNAc of the sialylated core 1 structure and ST8Sia VI can add sialic acid to C8 of the Neu5Ac sialylated core 1 structure, however these events appear to be uncommon. Thus, the major glycosylation abnormality of MUC1 in cancer results in the exposure of normally cryptic core Tn (GalNAc), and T (Gal  $\beta$  1-3 GalNAc) glycans, and the formation of the sialyl T (Neu5Ac $\alpha$ 2,3Gal $\beta$ 1,3GalNAc), and sialyl Tn (Neu5Ac $\alpha$ 2-6 GalNAc) glycans (Brockhausen I. *et al.* 1995, Lloyds K. *et al.* 1996, Burchell J. M. *et al.* 1999, Sewell R. *et al.* 2006) (fig 1.12).

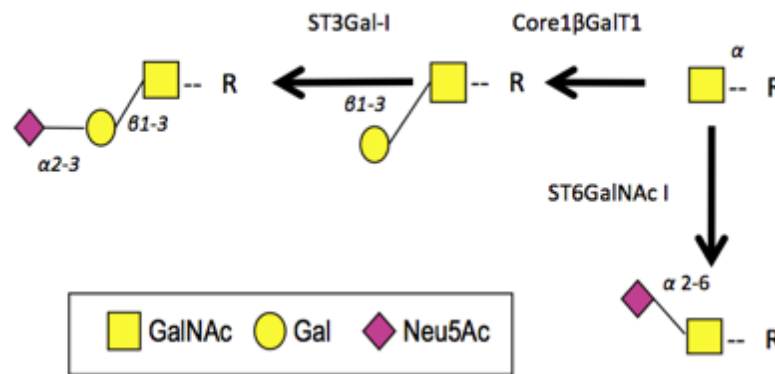
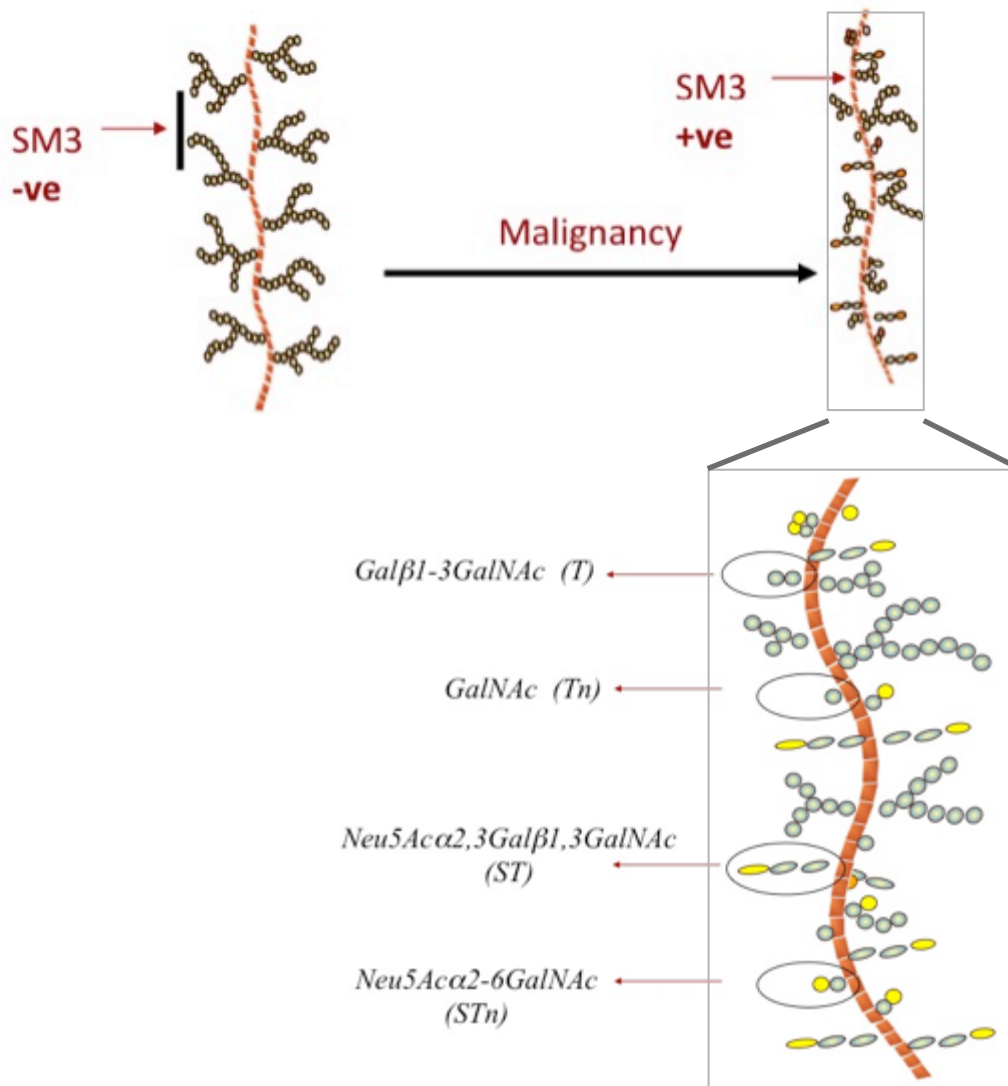


Figure 1.12 Pathways of O-glycosylation of MUC1 in mammary carcinomas

Sialylation of the core 2 tetrasaccharide can also occur preventing further extension of the O-glycans, and fucosylation of shortened sialylated core 2 can give rise to sLe<sup>x</sup> which is the minimal recognition motif of many selectins (Ley K. 2003). Although this form of glycosylation has not yet been proven to be present on MUC1, it is found in breast cancer (Julien S. *et al.* 2011).

The frequency of aberrant glycosylation of MUC1 in breast carcinomas appears to be a common event, as more than 90% of tumours stain strongly when using the monoclonal antibody SM3, which recognises the polypeptide PDTRP motif within the core protein of MUC1. However, SM3 does not react with MUC1 in normal resting breast due to the core 2 branch masking the polypeptide PDTRP motif recognized by SM3 (fig 1.13) (Dalziel M. *et al.* 2001, Mungul A. *et al.* 2004).



**Figure 1.13** *MUC1 associated O-linked glycan changes in breast cancer.*

*Schematic illustration of change commonly occurring (>90%) to O-linked glycans attached to MUC1 in breast cancer. Aberrant changes to the glycostructure of MUC1 mainly result in: GalNAc (Tn), Neu5Acα2,6GalNAc (Sialyl Tn), Galβ1,3GalNAc (T) and Neu5Acα2,3Galβ1,3GalNAc (sialyl T).*

### **1.5.2 Impact of aberrant glycosylation on the function of glycan carrying molecules**

The functional impact that altered glycosylation can deliver to cancer is well represented by the change leading to the increase in N-glycan elongation due to increased activity of N-acetylglucosaminyltransferase V (Mgat5). Increased Mgat5 expression is regulated by oncogene expression and is found to lead to β1,6GlcNAc



branching and sialylation (Yamashita K. *et al.* 1984, Hakomori S. 1996, Brockhausen I. *et al.* 1988). Furthermore, studies have shown that Mgat5 is associated with enhanced cell and tumour invasiveness (Yamamoto H. *et al.* 2000, Guo H. B. *et al.* 2007), and interestingly in a PyMT mouse model where the Mgat5 gene was knocked down, tumour progression and metastasis was diminished (Granovsky M. *et al.* 2000). In other studies, N-glycan elongation was found to affect selectin binding in both normal leukocytes and tumour cells, therefore highlighting a strong association between altered N-glycan elongation and metastasis (Zipin A. *et al.* 2004, Dennis J. W. 1987). Major carriers of N-glycans structures such as the group of surface receptors integrins appear to directly contribute to the cancer cell dissemination during metastasis (Rambaruth N. D. S. and Dwek M. V. 2011). Increases in the  $\beta$ 1,6GlcNAc structure on the  $\alpha$ 5 $\beta$  integrin subunit are found to increase adhesion to fibronectin, affecting cell-cell, and cell-matrix interactions promoting cell motility and invasiveness (Hakomori S. 1996).

Aberrant glycosylation of sialic acid-bearing glycosphingolipids (Ganglioside) can be used as a further example to highlight the variety of glycosylation changes found in cancer. In a variety of brain tumours, ganglioside glycosylation is markedly altered, influencing tumour growth and progression through modulation of adhesion, migration and angiogenesis (Hamasaki H. *et al.* 1999). The sialyltransferase ST6GalNAcV, which sialylates GM3 and GM2 $\alpha$ , is reduced in brain tumours, contributing to the increased expression of the GM3 and GM2 $\alpha$  structures, and correlates with increased invasiveness (Kroes R. A. *et al.* 2010). However, expression in breast cancer of ST6GalNAcV, which is normally restricted to the brain, is associated with brain metastasis by enhancing the adhesion of breast cancer cells to brain endothelium and their passage through the blood-brain barrier (Bos P. D. *et al.* 2009). Interestingly, the GM3 ganglioside structure has also been shown to inhibit NK cells responses, possibly mediated by antigen presenting cells (Grayson G. and Landisch S. 1992, Park J. E. *et al.* 2008).

As discussed in the earlier section (1.4), mucins such as MUC1 that are expressed by cancer cells including breast, express short O-linked glycans, and the most common are Thomsen-nouvelle (Tn), Thomsen-Friedenreich (T), and their sialylated version STn and ST. Moreover, Tn, STn and T have long been regarded as tumour-

associated carbohydrate antigens and ST is not normally found on mucins. Glycosylation changes of mucins mostly results from the modification of the expression of glycosyltransferase genes, with the level of expression of some sialyltransferases being proposed as a prognostic marker for the follow-up of breast cancer patients (Hebbar M. *et al.* 2003, Recchi M. A. *et al.* 1998). MUC1 carrying the tumour-specific glycans Tn and STn has been found to interact with antigen presenting cells such as macrophages and DC, through the macrophage galactose-type C-type lectin (MGL) in a  $\text{Ca}^{+2}$ -dependent manner (Napoletano C. *et al.* 2007, and personal communication of unpublished data). C-type lectins have been shown to participate in the detection of other tumour associated carbohydrate structures (Van Gisberger K. P. *et al.* 2005). Lectin-like receptors can take up antigen, and their cytoplasmic tail contains the C-type lectin signalling or internalization motif for antigen processing (Cambi A. *et al.* 2005), so it seems reasonable to propose that tumour-associated Tn and STn glycans carried on MUC1 can influence the balance between tolerance and immunity.

One of the most common tumour-associated carbohydrate antigens on MUC1 is sialyl T (Burchell J. M *et al.* 2001, Adamczyk B. *et al.* 2012). Interestingly, within the haemopoietic system the vast majority of cells express variable levels of sialic-acid-binding Ig-like lectins (Siglecs) (Cao H. and Crocker P. R. 2011). Siglecs are transmembrane molecules, and in humans this family of cell surface receptors is comprised of at least 16 different members. The vast majority of Siglecs contain inhibitory signalling motifs named immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails that mostly mediate inhibitory signalling (Avril T. *et al.* 2006, Crocker P. R. *et al.* 2007). The tumour-associated sialyl T on MUC1, contain sialic acids in an  $\alpha$ -2,3 linkage to Gal, which is the preferred linkage of several Siglecs (see table 1.5). Indeed MUC1 has been shown to bind to Siglec 1 (Nath D. 2001), and Siglec 4, which is not expressed by haematopoietic cells but by neurons (Swanson B. J. *et al.* 2007). However, Siglec 1 does not contain an ITIM motif but it is reasonable to assume that other Siglec may bind MUC1-ST, and unpublished data from our lab shows that MUC1 sialyl T can bind Siglec 3 (Dr. Beatson personal communication). Thus, it can be hypothesized that MUC1-ST via binding of Siglecs may transmit an inhibitory signal to the immune system.



**Table 1.5** Expression, function and preferred ligands of known human-related sialic-acid-binding immunoglobulin-like lectins

<b><i>Siglec</i></b>	<b><i>Cellular expression</i></b>	<b><i>Known functions</i></b>	<b><i>Preferred glycan</i></b>	<b><i>ITIM</i></b>
Human Siglec-1 (CD169)	Macrophages		$\alpha$ -2,3-linked sialic acid	No
Human Siglec-2 (CD22)	B cells	Mediate B cells interaction	$\alpha$ -2,6-linked sialic acid	Yes
Human Siglec-3 (CD33)	Myeloid progenitors, monocytes	Inhibition of Ca <sup>2+</sup> flux, cell growth and apoptosis. Release of cytokines	$\alpha$ -2,6-linked sialic acid	Yes
Human Siglec-4	Oligodendrocytes Schwann cells	Role in myelin maintenance, inhibitor of axonal growth Alt.	$\alpha$ -2,3-linked sialic acid	Yes
Human Siglec-5	Neutrophils, B cells	Increased respiratory burst in neutrophils and uptake of <i>Neisseria meningitidis</i>	$\alpha$ -2,6- and $\alpha$ -2,3-linked sialic acids	Yes
Human Siglec-6	Trophoblasts and B cells		Sialyl Tn structures	Yes
Human Siglec-7	Natural killer cells, minor subset of CD8 T cells, monocytes	Inhibition of cytotoxicity and T-cell receptor signaling	$\alpha$ -2,8-linked sialic acid, internal $\alpha$ -2,6-linked sialic acid; weaker to $\alpha$ -2,3- and $\alpha$ -2,6-linked sialic acids.	Yes
Human Siglec-8	Eosinophils, basophils	Induction of apoptosis	6'-sulphated sialyl Lewis X	Yes
Human Siglec-9	Neutrophils, monocytes, conventional dendritic cells	Inhibition of T-cell receptor-dependent signaling	$\alpha$ -2,6- and $\alpha$ -2,3-linked sialic acids	Yes
Human Siglec-10	Natural killer-like cells, B cells, monocytes and eosinophils	Binds CD24 and proposed to reduce inflammation in response to tissue damage	$\alpha$ -2,6- and $\alpha$ -2,3-linked sialic acids	Yes
Human Siglec-11	Macrophages especially microglia	Suppression of production of pro-inflammatory mediators and phagocytosis by microglia.	$\alpha$ -2,8-linked sialic acids	Yes
Human Siglec-12	Macrophages			Yes
Human Siglec-13	Eosinophil			Yes
Human Siglec-14	Neutrophils and monocytes		$\alpha$ -2,6- and $\alpha$ -2,3-linked sialic acids	No
Human Siglec-15	Tumour-associated macrophages	Enhance TGF- $\beta$ production	Sialyl Tn structures	No
Human Siglec-16	Macrophages			No

In addition to cancer-associated MUC1 potentially influencing tolerance and immunity via its interaction with the Siglecs, cancer-associated MUC1 has been found to be a natural ligand for galectin-3. Galectins have been shown to preferentially bind exposed galactose residues and the MUC1-galectin-3 interaction is via the binding of galectin-3 to the T antigen on MUC1 (Iurisci I. *et al.* 2000). Circulating galectin-3 in cancer patients is found to be elevated (Yu L. G. *et al.* 2007) and has been shown to promote the adhesion of breast cancer cells to endothelium by interacting with the T antigen carried by MUC1. This has been proposed to cause the redistribution of MUC1 on the cell surface and the exposure of smaller cell adhesion molecules allowing binding to the endothelium (see figure 1.14) (Iurisci I. *et al.* 2000).

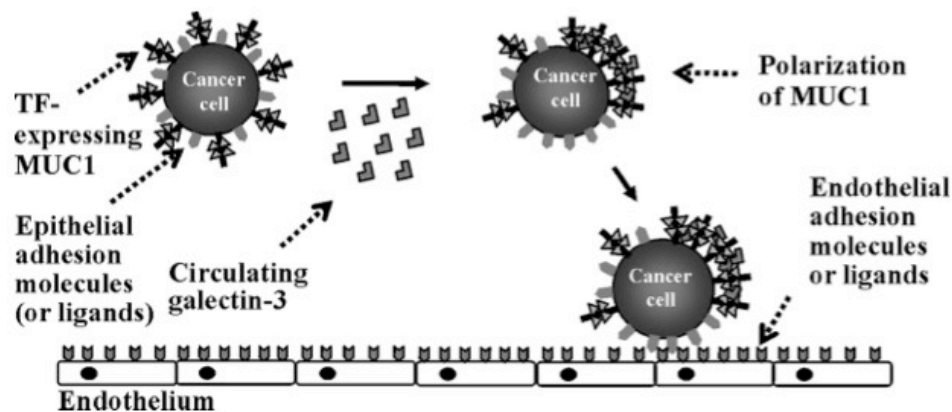


Figure 1.14 Proposed action of galectin-3-MUC1 interaction

(Taken from Lu-Gang Y. *et al.* 2007).

MUC1 has been found to interact with other receptor families such as the erbB family, especially EGFR. This enhances cancer cell proliferation through activation or inhibition of downstream signalling, and /or by suppressing the induction of apoptosis. As MUC1's expression is dramatically increased in cancer downstream signalling may be altered. In addition the aberrant glycosylation of the extracellular domain could affect the intracellular signalling by MUC1. For example when galectin-3 binds the T glycan on EGFR and/or MUC1 it can promote cellular proliferation (Merlin J. *et al.* 2011).

Changes in glycosylation seen in cancer may alter the length of time glycoproteins are retained on the cell surface, which will affect their function (Partridge E. A. *et al.* 2004, Hernandez J. D. *et al.* 2006). It is not clear if the aberrant glycosylation affects the retention on the cell surface of MUC1, but shorter side chains may aid protease cleavage of MUC1 from the cell surface.

The abnormal expression of sLe<sup>x</sup> functioning as a selectin ligand in cancer cells has been proposed to contribute to extravasation, mimicking a molecular mechanism involved in leukocyte extravasation mainly binding to E-selectin (Barthel S. R. *et al.* 2007, Laubli H. and Borsig L. 2010). Indeed in many cancers, but in particular colorectal, enhanced expression of sLe<sup>x</sup> and sLe<sup>a</sup> is seen, and these are closely associated with haematogenous metastasis (Ono M. and Hakomori S. 2004, Kannagi R. *et al.* 2004). Although in breast cancer sLe<sup>x</sup> expression has been associated with ER-negative status, lymph node involvement, and high grade of breast tumours, its correlation with bone metastasis, where E-selectin is constitutively expressed, was only seen in ER positive tumours (Julien S. *et al.* 2011).

## **1.6 Mechanisms attributed to the altered O-glycan profiles**

### ***Changes in expression of glycosyltransferases***

Of the glycosyltransferase enzymes involved in the core 1 to core 2 conversion of O-glycans added to MUC1, ST3Gal-I and C2GnT1 appear to play an important role by competing with each other for the same substrate Gal $\beta$ 1-3GalNAc in the Golgi apparatus. C2GnT1 regulates the glycosylation pattern found in normal mammary epithelium, and over-expression of ST3Gal-1 leads to the altered glycosylation pattern commonly found in breast cancer. The competition between those two glycosyltransferases has been demonstrated in cell lines (Mungul A. *et al.* 2004, Dalziel M. *et al.* 2001). ST3Gal-I has been found to be elevated in breast carcinomas, and its increased expression is correlated with tumour grade (Burchell J. *et al.* 1999). Expression of C2GnT1 was found to be low or absent in around 50% of breast cancer cell lines and carcinomas (Burchell J. *et al.* 1999, Whitehouse C. *et al.* 1997, Brockhausen I. *et al.* 1995), making ST3Gal-1 the more dominant enzyme in cancer.

In further support of the competition between ST3Gal-I and C2GnT1, a correlation has been observed between the level of mRNA expression encoding ST3Gal-I and the intensity of staining seen with the antibody SM3 (Burchell J. *et al.* 1999, Dalziel M. *et al.* 2001).

Like all glycosyltransferases the ST3Gal-I sialyltransferase has a type II transmembrane topology, and ST3Gal-I localizes to the Golgi body, where it overlaps to a certain degree with C2GnT1 (Whitehouse C. *et al.* 1997, Takashima S. 2008). It is composed of a short N-terminal cytoplasmic tail, a transmembrane domain, a stem region and a catalytic domain, with the whole protein being 337 amino acids in length (Fig 1.15). As with the other known sialyltransferases, ST3Gal-I contains highly conserved motifs called sialyl motifs L (Long), S (Short), III, and VS (Very Short) (Drickamer K. 1993, Jeanneau C. *et al.* 2004, Geremia R. A. *et al.* 1997). Sialyl motif L is characterized by a 55 amino acid region in the centre of the protein, involved in the binding of a donor substrate, CMP-Sia (Datta A. K. and Paulson J. C. 1995). Sialyl motif S is located in the C-terminal region and is 28 amino acid long, and has been found to be involved in the binding of both the donor and the acceptor substrate (Datta A. K. *et al.* 1998). Sialyl motif VS is located to the C-terminal region, and this motif is involved in its catalytic activity (Jeanneau C. *et al.* 2004).

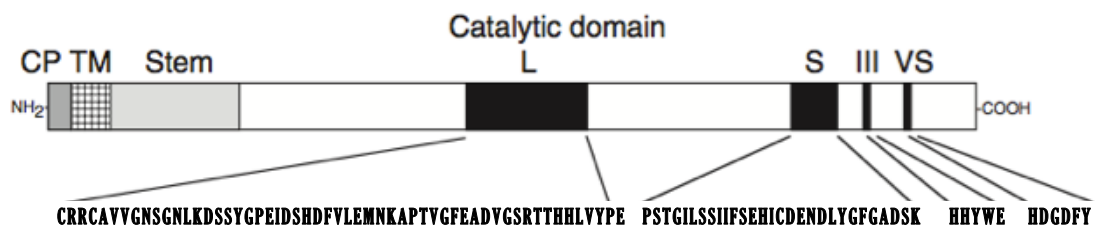


Figure 1.15 Structure of ST3Gal-I

CP, Cytoplasmic tail; TM, transmembrane domain; Stem, stem region; L, sialyl motif L; S, sialyl motif S; III, sialyl motif III; VS, sialyl motif VS.

Amino acid sequences have revealed that in the mouse and human genome ST3Gal-I shares about 50% similarity with ST3Gal-II, but relatively less (about 30%) with the other members of the same family, ST3Gal-III, IV, V and VI (Takashima S. 2008). In mice, the ST3Gal-I gene is located on chromosome 15 (15D2), and in humans is located on chromosome 8 (8q24.2) (Takashima S. *et al.* 2002). Knockout of the gene

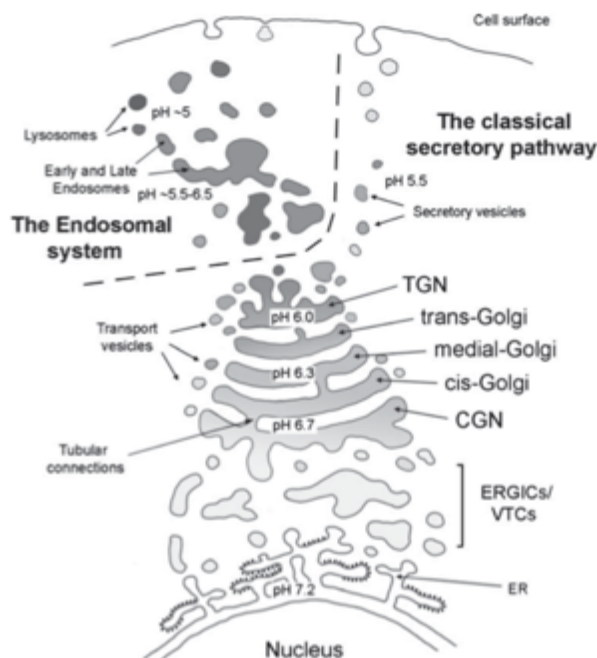
in mice showed that this glycosyltransferase plays an important role in the sialylation of core 1 O-glycans in T lymphocytes, which is involved in the survival of CD8<sup>+</sup> T cells by making them more resistant to apoptosis (Priatel J. J. *et al.* 2000). However, a more recent study by Kao and colleagues found that CD8-induced cell death is not markedly influenced by ST3Gal-I deficiency, and that this deficiency has no detectable impact on CD8 binding to multimeric peptide/MHC class I ligands at any stage of thymocyte development (Kao C. *et al.* 2006). Human ST3Gal-I has been found to have five potential N-glycosylation sites, which are not necessary for enzyme activity, but which appear to contribute to the proper folding and trafficking of the enzyme (Jeanneau C. *et al.* 2004).

In breast cancer cell lines, Cyclooxygenase-2 (COX-2) was shown to increase expression of ST3Gal-I, working through the prostanoid prostaglandin E2 (PGE2) (Sproviero D. *et al.* 2012). Importantly, COX-2 is overexpressed by about 40% of invasive breast carcinoma (Ristimäki A. *et al.* 2002), and is associated with markers of poor prognosis, such as HER2 and aromatase overexpression (Brueggemeier R. W. *et al.* 1999, Ranger G. S. *et al.* 2004). In the colon carcinoma cell line HT-29, the tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) can enhance ST3Gal-I expression through NF $\kappa$ B binding sites (Higai K. *et al.* 2006), and in porcine kidney cells, transcriptional activation of ST3Gal-I was shown to be induced by tumour growth factor  $\beta$ 1 (TGF- $\beta$ 1) (Son S. W. *et al.* 2011). Thus, suggesting that the malignant increased expression of ST3Gal-I may be caused by several mechanisms.

Sialylation of the first sugar (GalNAc) in a  $\alpha$ 2,6 linkage is an event, which has been found on 25-30% of breast carcinomas (Miles D. *et al.* 1994, Imai J. *et al.* 2001). ST6GalNAc-I and ST6GalNAc-II, have been identified as the sialyltransferases which are able to add sialic acid in  $\alpha$ 2,6 linkage to GalNAc which is linked to serine or threonine, thus creating STn (Ikehara Y. *et al.* 1999). However, when the transcription of ST6GalNAc-I was turned on a correlation was found with STn expression in primary breast cancer (Sewell R. *et al.* 2006), whereas no correlation was seen with ST6GalNAc-II expression. Because, ST6GalNAc-I mRNA is not detectable in normal human mammary glands, in the 25-30% of breast cancers where it is seen, a molecular switch must occur to switch on the transcription of this enzyme.

### ***Changes in Golgi pH***

Although, it is still debatable whether the Golgi apparatus is a distinct organelle or just an extension of the endoplasmic reticulum, it remains the most important site for several post-synthetic modifications, including O-linked glycosylation (Varki A. 1998, Brockhausen I. 1999). The lumen of the Golgi apparatus in normal cells is weakly acidic, gradually decreasing from neutral in the endoplasmic reticulum (pH 7.0), to acidic in the trans-Golgi network (pH 6.0-5.5) (Fig 1.16) (Weisz O. A. 2003, Paroutis P *et al.* 2004). Sialic acid terminal glycosylation also requires acidic conditions in the Golgi lumen (Thorens B. and Vassalli P. 1986), and acidic pH is known to be necessary for the optimal activity and correct localization of the glycosyltransferases responsible for sialic acid terminal glycosylation (Bretz R. *et al.* 1980, Varki A. 1998, Axelsson M. A. *et al.* 2001). In recent years, abnormally alkaline Golgi pH has been reported in many cancer cells and cancer cell types, including those derived from breast, and this appears to correlates with T antigen expression (Rivinoja A. *et al.* 2006).



**Figure 1.16** *The secretory pathway and the endosomal system.*

*Golgi apparatus composed of the cis-Golgi network (CGN), and the Golgi stack and the trans-Golgi network (TGN). The Golgi stack can be further divided into cis-, medial-, and trans-Golgi. (Taken from Rivinoja A. *et al.* 2006)*

### ***Relocation of pGalNAc-Ts***

The process that regulates the addition of O-linked glycans onto mucins is complex, and fine-tuning of the expression of particular GalNAc-transferases within the Golgi is extremely important. Given the fact that several substrates can be modified by several GalNAc-Ts, and as competition between GalNAc-Ts leads to different patterns of glycosylation (Bennett E. P. *et al.* 1999, Hanisch F. G. *et al.* 2001, Perrine C. L. *et al.* 2009, Kato K. *et al.* 2001), their localized expression provides a mechanism for regulating their actions. While GalNAc-Ts in cells and normal tissues are present in the Golgi apparatus (Mandel U. *et al.* 1999), a recent finding appears to indicate that their localization is subject to the cellular physiological state, and under certain conditions can be found within the ER (Gill D. J. *et al.* 2011). Src activation has been shown to result in the selective redistribution of Golgi-localized GalNAc-Ts to the ER, and that the redistribution is dependent on the retrograde transport associated with the COPI protein (Gill D. J. *et al.* 2011). The relocation of GalNAc-Ts to the ER resulted in increased duration of availability to potential O-glycosylation substrates without competition, which led to an increased density of sites with GalNAc attached. This may result in many substrates for the core 1 enzyme being generated, and indeed, in their experimental system, increased expression of Tn and T was observed (Gill D. J. *et al.* 2011).

### ***Mutations in the Cosmc gene***

Although the altered O-glycan profile of tumour-associated MUC1 can be partly explained by the enhanced or reduced expression of certain glycosyltransferases, and changes in pH can affect optimal activity and correct localization of glycosyltransferases, other cancer-specific changes/mutation may contribute to the tumour-associated glycan profile. For example, another reported explanation for the increase in expression of two tumour-associated carbohydrate antigens, Tn and Sialyl Tn, is the loss-of function/mutation in the *Cosmc* gene, which code for the chaperone required for the  $\beta$ 1,3 galactosyltransferase, the core 1 enzyme (Ju T. *et al.* 2008). Loss of activity of *Cosmc* results in incorrect folding of the  $\beta$ 1,3 galactosyltransferase resulting in loss of its activity. As it is the only glycosyltransferase responsible for the formation of core 1, mutations in *Cosmc*

result in loss of core 1 and expression of Tn or STn if ST6GalNAc-I is expressed by the cell. However, the loss-of function of the *Cosmc* gene will result not only in the expression of the tumour-associated carbohydrate antigens Tn and Sialyl Tn on MUC1, but on all the O-linked glycoproteins. Thus cellular loss-of function of *Cosmc*, would result in the O-linked glycans expressed by the cells being limited to the carbohydrate GalNAc and its sialylated form if ST6GalNAc-I is expressed. However, our laboratory recently investigated co-expression of the carbohydrate T with Tn on MUC1 in mammary carcinomas (see appendix I) and found that 100% of the investigated tumours expressed both types of glycans. If the loss-of function of *Cosmc* contributed to the altered O-glycan profile in these tumours, Tn and T would not have been observed together. Thus, the results clearly indicate that the altered O-glycan profile of these breast cancers did not result from loss-of function of *Cosmc*.

## 1.7 Tumour immunology

In 2000 Hanahan and Weinberg described cancer as requiring six steps for its formation; an insensitivity to anti-growth signals, avoiding apoptosis, sustained angiogenesis, self sufficiency in growth signals, limitless replication potential, and tissue invasion and metastasis (Hanahan D. and Weinberg R. A. 2000). Interestingly since their publication in 2000, a substantial body of evidence have lead the authors to a conceptual progression, and recently they have added further steps including evading immune destruction (fig 1.17) (Hanahan D. and Weinberg R. A. 2011).



*Figure 1.17  
Proposed hallmarks of cancer*

*(Taken from Hanahan D. and Weinberg R. A. 2011).*



Evidence that the immune system is able to prevent or control tumour formation has been accumulating in recent years, and includes:

- 1) Immunocompromised individuals have an increased occurrence of certain cancers (Vajdic C. M. and van Leeuwen M. T. 2009) and in individuals who had an organ transplant, correlation was found between tumours and immunosuppressive agents (Penn I. 2000).
- 2) Patients who have tumours that are heavily infiltrated with CTLs and NK cells have a better prognosis than those that lack such abundant killer lymphocytes (Pages F. *et al.* 2010, Nelson B. H. 2008).
- 3) It is well documented that many tumours lose expression of MHC class I (Ochsenbein A. F. 2002, Garrido F. *et al.* 1997).
- 4) Experiments which used genetically engineered mice deficient for various components of the immune system, helped to clarify that both the innate and adaptive cellular arms of the immune system contribute to immune surveillance and thus tumour eradication (Kim R. *et al.* 2007, Teng M. W. *et al.* 2008).

Following from the fact that the immune system is sometimes capable of eradicating or controlling tumour growth, the immune system must be provided with means of recognising malignancies by recognizing either tumours-specific antigens (TSA), which are only present on tumours cells, or tumours-associated antigens (TAA), which are antigens present mostly on tumour cells but also on some normal cells. TSAs are the ideal targets for cancer immunotherapy because of their specificity. They are largely composed of mutant proteins caused by somatic mutations in the original sequence of the protein chromosome relocations or changes in protein glycosylation. A major advantage of targeting TSAs is that many of these proteins, such as the oncogene RAS and BRAF, have been demonstrated to be essential for tumorigenesis and cancer progression (Bendle G. M. *et al.* 2005, Kudchadkar R. *et al.* 2012). In contrast, TAAs are commonly expressed on tumours with the same histology and are often shared among tumours of different origin. A major limitation of targeting TAAs is that they are weakly immunogenic due to the tolerance to self-antigens acquired by the immune system in its developmental stage (Schieteringer A, *et al.* 2008).

Novellino *et al.* in 2005, classified the TAA into 7 different groups:

Cancer germ line genes (e.g. MAGE).

Novel antigens encoded by tumour specific transcripts (e.g. TRP2).

Viral antigens (e.g. EBV).

Antigens resulting from altered post-translational modification (PTM) (e.g. MUC1).

Antigens resulting from mutation (e.g. Bcr-abl).

Differentiation antigens (e.g. CEA).

Over expressed tumour antigens (e.g. HER2, MUC1).

According to this classification MUC1 falls into two groups, as expression levels increase markedly in the cancer context (about 100 fold) and MUC1 O-linked glycosylation is changed. Thus, MUC1 glycan changes in cancer ought to allow an effective immunological response by enabling the immune system to effectively differentiate between healthy and cancerous cells, and to destroy the tumour. However, the ability of MUC1 positive tumours to proliferate and metastasise shows that the immune system is unable to destroy those tumours, perhaps because the tumour cells have developed ways of escaping an immune response.

## **1.8 Immune evasion**

Immune evasion can be defined as when the immune system is no longer able to recognise the tumour or is handicapped in some way by another factor associated or induced by transformation. Transformed cells may have developed many ways of escaping an immune response, specifically, a Th1, cellular response. For example:

- The tumour may have simply outgrown the immune system, and is proliferating at a rate, which is too great for an effective response.
- There is an access problem for the cells as tumours are often dense, with a necrotic core and a poor blood supply (although this is also problematic for the tumour).
- There is tolerance (anergy) of T cells to the tumour antigen (e.g. due to no co-stimulation by modulated APCs).

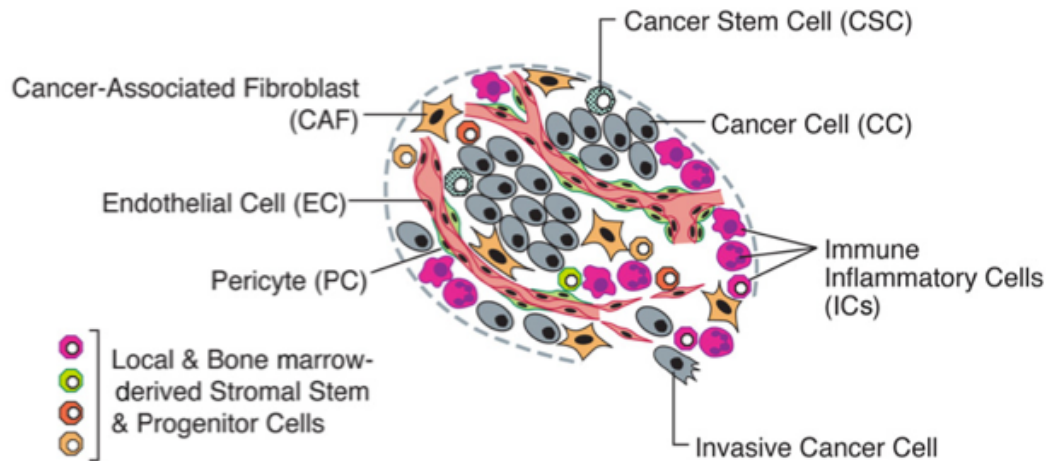
- There is a proliferation of Tregs and myeloid-derived suppressor cells (MDSCs). These cells dampen down Th1 and Th2 responses and so hamper a response.
- The transformed cells either directly or indirectly confers immunoprivilege (e.g. IL-10) or immune subversion (e.g. IL-6 and the production of Th17 cells).
- There can be further transformation of the tumour (e.g. loss of antigen, loss of TAP which is critical for MHC class 1 processing, increase in cell surface FasL, loss of MHC altogether).

Many of these factors come together in the local tumour microenvironment the location where effector immune cells must operate (Pawelec G. 2004; Rivoltini L. *et al.* 2005). Transplantation experiments have shown that the transplantation of cancer cells originating in immunodeficient mice are generally inefficient at initiating secondary tumours in syngeneic immunocompetent hosts, but cancer cells originating from immunocompetent hosts are equally efficient at initiating secondary tumours in both immunodeficient and immunocompetent hosts (Kim R. *et al.* 2007, Teng M. W. *et al.* 2008). A proposed interpretation of the data from transplantation experiments was that immunogenic cancer cells are routinely eliminated in immunocompetent hosts, and only weakly immunogenic variants grow and generate solid tumours, and those weakly immunogenic cells can thereafter colonize both immunodeficient and immunocompetent hosts. Instead, when cancer cells are generated in immunodeficient hosts, the immunogenic cells are not selectively depleted, and when cells from those tumours are transplanted into syngeneic recipients, the immunogenic cancer cells are rejected from the immune systems of their secondary hosts (Smyth M. J. *et al.* 2006).

## **1.9 Tumour microenvironment**

Tumours are composed not only of cancer cells but also other cell types constituting the stroma, and as much as 50% of the total tumour mass can be composed of leukocytes in some invasive breast carcinomas (Eiro N. and Vizoso J. F. 2012). Within the tumour microenvironment, non-transformed cells, such as endothelial

cells, fibroblasts, other mesenchymal cells, and cells of the innate and adaptive immune system (fig 1.18), interact with malignant tumour cells to form a dynamic tumour microenvironment in which the non-transformed cells exert both positive and negative effects on the growth and spread of cancer cells (Alexander S. and Friedl P. 2012).



*Figure 1.18 Example of tumour microenvironment*  
(Taken from Hanahan D. and Weinberg R. A. 2011).

During the early stages of cancer its microenvironment appears to be of particular importance, as carcinogenesis can result from the inflammation associated with a variety of chronic infections e.g. ulcerative colitis (Kuraishy A. *et al.* 2011). Historically, leukocytes infiltrating the tumour have been considered to be the manifestation of an intrinsic defence mechanism against developing tumours (Johnson J. P. *et al.* 1989, Lin E. Y. and Pollard J. W. 2004). This is supported by an increasing body of evidence, that shows that specific cell populations, from both the innate and adaptive immune systems, interact with developing tumours and frequently contribute to the arrest of tumour growth as seen by tumour regression in animal models and cancer patients (Draghiciu O. *et al.* 2011). However, evidence also indicates that leukocytes can also promote angiogenesis, growth and invasion of tumours (Coussens L. M. and Werb Z. 2002, Daniel D. *et al.* 2005). This is thought to be the result of leukocytes secreting cytokines, growth factors, chemokines and proteases such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), chemokine ligand 12 and IL8 (Robinson S. C. and Coussens L. M.

2005, Benelli R. *et al.* 2006), which stimulate proliferation and invasiveness of cancer cells. The balance of cellular and secretory-product interactions within the early tumour microenvironment appears to determine whether the tumour mass regress or grows.

Tumour progression and spread, facilitated by cells of the immune system, has also been proposed to be the result of selection of tumour cells intrinsically capable of escaping immune recognition (Schreiber R. D. *et al.* 2011), and any late stage antitumor immune response must occur in a mix of transformed cells, stroma, fibroblasts, necrotic and apoptotic cells, anergic infiltrating leukocytes and immunosuppressive factors. Many of these factors inspire alternatively activated macrophages and DCs, ineffective monocyte differentiation and function, Treg induction, B cell class switching, Th17 production, NK and neutrophil inactivity. It is an extremely difficult environment in which to mount an immune attack (Petrulio C. A. *et al.* 2006; Mantovani A. *et al.* 2008).

The effects of intratumoral cytokines and tumour cell biology may affect the ability of primary breast cancer patients to mount natural immune responses. A recent study found that about 40% of breast cancer patients (n=59) had tumour specific CTLs, and this correlated with highly differentiated tumours, ER expression, and low proliferative activity, which led to an overall reduction in mortality. High tumour differentiation also correlated with high local IFN $\alpha$  and low local TGF $\beta$ 1. The presence of tumour specific B cell responses correlated positively with advanced tumour stage, increased TGF $\beta$ 1, reduced IFN $\alpha$  and absence of CTL responses. Thus, CTL responses in breast cancer appear to be associated with better prognosis, (Domschke C. *et al.* 2009).

Antibody levels to MUC1 in breast cancer patients have been shown to correlate with improved survival (von Mensdorff-Pouilly S. *et al.* 1996, Blixt O. *et al.* 2011). In the study conducted by Blixt and colleagues, antibodies to aberrant glycoforms of MUC1 correlated with improved survival. Changes in glycosylation can induce humoral responses that play some role in controlling tumour growth. It is reasonable to assume that leukocytes infiltrating the tumours target MUC1, and can mount an

intrinsic defence mechanism against developing tumours, and that the tumour microenvironment can affect this intrinsic defence mechanism. A recent trial was carried out using MUC1-specific autologous CTLs induced *ex vivo*, followed by reinfusion to previously treated metastatic breast cancer patients. Results from this trial showed that CTLs generated from patients with high tumour burden had lower cytokine production and cytotoxicity against MCF-7 cells than those in remission. This adds to the idea that the tumour burden and environment plays a role in immunosuppression (Wright S. E. *et al.* 2009). However, the trial was small, with only four patients enrolled.

### **1.10 MUC1 in immunotherapy**

MUC1 has attracted a lot of interest as a potential target for immunotherapy against cancer because of three main characteristics. Firstly, because it is underglycosylated in cancer, cryptic peptide epitopes within the VNTR region are unmasked, enabling tumour-selective binding by several antibodies and autoantibodies (Girling A. *et al.* 1989, Tarp M. A. *et al.* 2007, Blixt O. *et al.* 2011). Secondly, its transcription is up regulated in many tumours, notably breast, ovary, pancreas and lung (Girling A. *et al.* 1989). Thirdly, in cancer cells, cell polarity is lost (Hilkens J. *et al.* 1984), which results in loss of MUC1 apical polarization and so this mucin is found over the entire cell surface. Immune responses to MUC1 have been identified in cancer patients, and CD8<sup>+</sup> T cells specific for MUC1 peptides can be present in the peripheral blood of some breast cancer patients (Correa I. *et al.* 2005), the presence of circulating antibodies directed to the aberrantly glycosylated MUC1 correlates with a better prognosis for breast cancer patients (von Mensdorff-Pouilly S. *et al.* 1996, Iurisci I. *et al.* 2000, Blixt O. *et al.* 2011) both indicating that the immune system is able to mount a response to aberrantly glycosylated MUC1.

Several forms of MUC1, including unglycosylated MUC1 TR peptides and MUC1 expressed in vaccinia virus, have been used in immunisation studies in both mice and humans. The different vaccines have in some cases induced humoral and cytotoxic immunity against MUC1 (Scholl S. M. *et al.* 2000, Soares M. *et al.* 2001, Quoix E. *et al.* 2011), and more recent trials showed improved survival in non small-cell lung

cancer patients when given in combination with first line chemotherapy (Quoix E. *et al.* 2011). However, the influence of glycan-specific MUC1 glycoproteins on immunity has not yet been fully determined, probably due to the non-availability of glycan specific MUC1 glycoproteins. To investigate whether any aberrant MUC1 glycans have immunological properties, our group (in collaboration with a European consortium and this project) have generated recombinant MUC1 glycoproteins carrying tumour-associated glycans, developed by expressing the extracellular part of human MUC1 fused with murine IgG Fc in CHO-K1 (Chinese-hamster ovary K1) cells, and in CHO IdID mutant cells with or without transfected glycosyltransferases.

### ***1.11 The known immunological effects to cancer associated MUC1 glycoforms***

Glycans are a common structure in the body, and MUC1 is a heavily glycosylated mucin (see section 1.4.3), however in a normal scenario, the glycans expressed on MUC1 would not come in contact with cellular component of the immune system as MUC1 is mainly expressed on the apical surface of cells. During tumourigenesis MUC1 apical expression is lost, and is expressed on the entire cell surface of the tumour cell. Interestingly, it is this aberrant, abundantly repeated and clustered nature of the glycans on MUC1 that resembles a pathogen associated molecular pattern (PAMP). These structures may therefore confer the same immune responses as seen against pathogens, with regards to both immunosuppression and stimulation.

#### ***MUC1-Tn***

Recombinant human MUC1-Tn peptides have been observed to bind to the C-type lectin MGL, expressed on macrophages and immature DCs (Saeland E. *et al.* 2007, Napoletano C. *et al.* 2007). The effect of this interaction is currently unknown, however MUC1-Tn peptides can be delivered to both class I and II pathways, suggesting cross-presentation is possible (Napoletano C. *et al.* 2007). Furthermore, some studies suggest that cleavage of glycoprotein by the immunoproteasome is achievable and so presentation of MUC1-Tn on both MHC class I as well as II is possible (Lakshminarayanan V. *et al.* 2012). In mice vaccinated with MUC1-Tn

glycopeptides, MUC1-Tn specific CTLs were seen (Xu J. *et al.* 2004; Stepensky D. *et al.* 2006). Predicted peptides within the MUC1 TR domain carrying GalNAc have been shown to bind MHC class I HLA-A 0201 (HLA-A2) molecules (Ninkovic T. *et al.* 2009). However, the engagement of lectins without danger signals to activate TLRs has the potential to induce tolerogenic DCs and induce T cell anergy (Cao H. and Crocker P. R. 2011).

### ***MUC1-STn***

The immunomodulatory role of STn-expressing mucins has been investigated for many years. They have been shown to inhibit natural killer cell cytotoxicity (Ogata S. *et al.* 1992; Zhang K. *et al.* 1997), and evidence is building for the immunomodulative effects of mucins expressing this glycan by increasing IL6 production from monocytes (Yokoigawa N. *et al.* 2005). As with MUC1-Tn, MUC1-STn peptide immunizations have been shown to elicit strong humoral responses in MUC1 Tg mice (Sorensen A. L. *et al.* 2006)

### ***MUC1-T***

The Thomsen-Friedenreich (T) antigen is frequently expressed in normal physiology as well as many cancers (Byrd J. C. and Bresalier R. S. 2004). MUC1 carrying the T antigen (MUC1-T) is known to interact with galectins, with much research focusing on galectin-3. For example as described in section 1.5.1, circulating galectin 3 can associate with MUC1 via T expressed on circulating tumour cells, which results in clustering of MUC1 thereby promoting cancer cell adhesion by revealing epithelial adhesion molecules (Yu L. G. *et al.* 2007).

Very interestingly, MUC1-T is present in the normal stomach, and *Helicobacter pylori* (*H. pylori*) has been shown to bind to this glycoform (Linden S. K. *et al.* 2009). Whether this is beneficial for the host or bacteria is unclear. However, *H. pylori*-infected individuals express anti-MUC1-T antibodies (Klaamas, K. *et al.* 2007), and it has been suggested that this is a result of epitope-spreading during inflammation.



## MUC1-ST

Using a transplantable tumour model in human MUC1 transgenic mice, it was observed that a murine mammary tumour transfected with human MUC1 carrying sialylated core 1-based O-glycans (ST) grow significantly faster than identical tumours transfected to carry MUC1 with core 2-based O-glycans. This difference can be seen in figure 1.19. As this effect was not observed in immunodeficient mice, the mechanism involved was thought to be immunological (Mungul, A. *et al.* 2004).

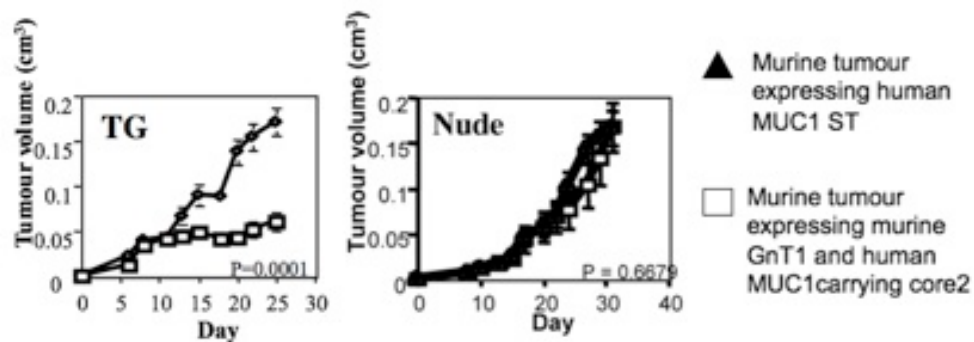


Figure 1.19 MUC1-ST tumour cells grow faster in MUC1 transgenic mice than MUC1-core 2 tumour cells.

E3 cells (410.4 murine mammary cell line transfected with human MUC1) and E3-core 2 cells (410.4 murine mammary cell line transfected with human MUC1 and murine C2GnT-I) were injected subcutaneously into MUC1 transgenic and nude mice. Tumour volume was assessed every 2/3 days. (n=20 for MUC1 transgenic, n=10 for nude)(Figure taken from Mungul *et al.* 2004).

Using recombinant human MUC1-ST (as described in Chapter 3) produced in CHO cells, Rughetti and colleagues showed that this glycoform affected the differentiation of monocyte-derived DCs. When matured in the presence of MUC1-ST these cells produced high levels of IL-10 and low levels of IL-12, whereas cells matured in the absence of MUC1-ST produced high IL-12 and low IL-10. In addition, monocyte-derived DCs treated for 24h with MUC1-ST were less able to stimulate lymphocyte proliferation in an allogenic mixed leukocyte reaction (MLR), and were less efficient at stimulating a recall response in T cells (Rughetti A. *et al.* 2005). Furthermore, MUC1 isolated from tumour cells (where the dominant glycan is likely to be MUC1-ST) has been seen to switch DCs to an IL-10<sup>high</sup>, IL-12<sup>low</sup> phenotype and thus trigger T cell anergy (Monti P. *et al.* 2004).

### ***Undefined tumour-associated MUC1 glycoforms***

In a mouse model of spontaneous pancreatic cancer expressing human MUC1, tumours expressed higher levels of cyclooxygenase-2 (COX-2) and indoleamine 2,3-dioxygenase (IDO) compared with tumours lacking MUC1, especially during early stages of tumour development. MUC1 expression correlated with an increased percentage of regulatory T cells and myeloid suppressor cells in the pancreatic tumour and tumour draining lymph nodes. (Tinder T. L. *et al.* 2008). Another group found that cancer-associated MUC1 attracts immature DCs through chemotaxis and subverts their function by negatively affecting their ability to stimulate type 1 helper T cell responses. MUC1 transgenic mice were shown to mount only a humoral response when immunized with tumour-associated MUC1 expressed in murine adenocarcinoma cells (Chen D. *et al.* 2003). A lower infiltrate of CD8 cells was seen in sialylated MUC1-expressing eyelid carcinomas (Uehara F. and Ohba N. 2002), and affinity-purified MUC1 from ascites was seen to inhibit T cell proliferation, which was reversed by the addition of exogenous IL-2 (Agrawal B. *et al.* 1998).

An understanding of the specific immunological effects of each tumour-associated MUC1 glycoform is just beginning. Evidence suggests that there are differences between the glycoforms and that there is an interaction with the immune system. This strongly suggests that different MUC1 glycoforms interact with different lectins, evoking a glycan-specific immune response. Lectins are known to interact with pathogens (e.g. several C-type lectins) but they also interact with other host cells (e.g. selectins and Siglecs). It is the interaction between lectins and specific tumour-associated MUC1 glycoforms that is believed to be the determining factor in eliciting and or suppressing specific immunological effects.

# ***Chapter 2***

## ***Materials and methods***

## ***Materials and methods used in chapter 3***

### ***2.1 Enterokinase cleavage of murine IgG2aFc domain***

Concentrated culture supernatant from cells (CHO K1, mutant CHO-I<sub>Δ</sub>1D with or without the glycosyltransferase ST6GalNAcI) transfected with MUC1-Ig (Fc region from mouse IgG2a fused to MUC1) was obtained from Prof T. Noll (Jülich). Supernatant was diluted with EK<sub>Max</sub> buffer (50 mM Tris/HCl, pH 8.0, 0.05% Tween 20 and 1 mM CaCl<sub>2</sub>) to yield a concentration of 0.6 mg/ml. 500 U EK (EK Max, Invitrogen, Cat No. E180-01) was added, and the mixture was incubated for 24 hours at 37°C (83 U/mg). A further 500 U was then added and the mix was incubated for a further 24 hours. The efficiency of the cleavage was assessed by silver and Alcain blue stains and the samples immediately purified.

### ***2.2 Anion exchange chromatography*** (Backstrom M. *et al.* 2003)

Enterokinase cleaved MUC1 from WT CHO cells were purified by anion exchange chromatography. A HiPrep 16/10QFF (GE Healthcare, Uppsala, Sweden Cat No 28-9365-43) sepharose ion exchange column was used. The column was stored at 4°C in 20% ethanol. The column, superloop (GE Healthcare, Uppsala, Sweden, Cat No 18-1113-82), waste collectors, fraction collection tubes and solutions were prepared on the AKTA purifier (AKTApurifier 10, GE Healthcare, Uppsala, Sweden, Cat No 28-4062-64). The solutions required were as follows: 'High salt solution' (2M NaCl), 'Low salt solution' (50 mM Tris-HCl pH 8.0), warmed and degassed deionized water (DW) and 20% ethanol in DW. Air bubbles were removed from system manually prior to a run. Degassed DW was run through column for 25 minutes at a rate of 2 ml/min with a maximum pressure of 0.35 MPa. Low salt solution was run through column for 25 minutes at a rate of 2 ml per minute with a maximum pressure of 0.35 MPa. The superloop was filled with sample manually (10-25 ml). The program was started.

*Outline of the programme:* The column was equilibrated to “low salt conditions” (50 mM Tris-HCl pH 8.0) for 3 column volumes, the sample was then loaded on to the column at 1 ml/min before the unbound material was washed out with low salt solution into the waste collector (1 column volume). The percentage of high salt solution (2 M NaCl) was gradually increased with elution fractions being collected every 3 ml (Fraction collector 901, GE Healthcare, Uppsala, Sweden Cat No 18-1118-97). The percentage of 2 M NaCl was increased from 0-7.5%, before being held at 7.5% for 2 column volumes – it is at this plateau that the IgG2aFc domain elutes. The percentage of 2 M NaCl then increased from 7.5% to 25% over 5 column volumes before increasing sharply to 100%. It is from the 7.5% to 25% 2 M NaCl that the rhMUC1-ST elutes. Due to the sticky nature of MUC1, the column was then deep cleaned according to manufacturers instructions.

*Deep cleaning of IEX:* 80 ml of 2 M NaCl was run through the column at 5ml/min (0.35 Mpa max pressure), followed by 50 ml degassed DW. 80 ml of 1 M NaOH was then run through the column, followed by 50 ml degassed DW, 80 ml 70% ethanol, again followed by 60 ml degassed DW. Finally, 80 ml 20% ethanol was run through before ending the programme and storing the column.

### **2.3 Affinity chromatography (MUC1 without glycans and Tn)**

Enterokinase cleaved MUC1 without glycans or carrying Tn secreted by CHO-IldID cells was purified by affinity chromatography.

*Column preparation:* CnBr activated sepharose 4B (Sigma) was used at a ratio of 1 g per 5 mg of Ab. Sepharose was washed in 1 mM HCl (200 ml/g) using a sintered glass filter, before adding the 5E5 antibody for purification of MUC1-Tn or HMFG1 antibody for purification of MUC1 without glycans were previously dialysed O/N in 0.1 M NaHCO<sub>3</sub>, 0.5 M NaCl pH 8.0 (coupling buffer). Ab/sepharose mixture was incubated for 2 hours at room temperature, before being washed with 200 ml/g of coupling buffer. Non-reacted groups were blocked in 5 ml/g of 1M ethanolamine (pH 8.0) for 2 hours at room temperature, before activation with low pH buffer (0.1 M Na acetate, 0.5 M NaCl pH4.0) follow by coupling buffer. The sepharose/Abs gel was

then placed into a disposable plastic column (Thermo scientific cat no: 29924) and stored at 4°C.

*Purification:* The procedure was performed at 4°C. Enterokinase cleaved MUC1 without glycans or Tn from CHO-IldID cells was added to CnBr/Ab column (flow rate 1ml/5 minutes), washed with Phosphate Buffer Saline (PBS) (200 ml/mg), before elution with 0.1 M glycine (pH 2.5) in 500 µl fractions, which were collected into eppendorf tube containing 100 µl of 1 M TRIS (pH 9.0). Concentrations of fractions was determined by enzyme-linked immunosorbent assay (ELISA) (see sections 2.11 and 2.12).

## **2.4 Neuraminidase treatment of purified recombinant MUC1-ST**

Recombinant MUC1-ST was dialysed in 6-8 kDa membrane (Spectropor) against 5 litres of sodium acetate buffer (50 mM NaAc pH 6.0, 4mM CaCl<sub>2</sub>, 0.02% azide) overnight at 4°C. The next day, the appropriate amount of neuraminidase beads (Sigma Cat No N5254) was washed twice in sodium acetate buffer – 0.15 U of beads per mg of MUC1-ST. The dialysed MUC1-ST was mixed with the washed neuraminidase beads in an Eppendorf and rotated overnight at room temperature (RT). The mix was then spun at 100 G in a microcentrifuge, with the supernatant being removed to a new tube and 10 µl taken and either a) checked on an Alcian blue stain b) checked on a Western using HMFG1, c) checked on a lectin ELISA against the starting material. If the treatment was successful, the MUC1-T was further dialysed in 8-10 kDa membrane (SpectraPor Cat No 132645) against 5 litres of DW.

## **2.5 SDS-PAGE (Polyacrylamide gel electrophoresis)**

*Gradient Gels (commercial):* NuPAGE Novex 4-12% Bis-Tris Gel 1.0 mm, 10 well (Invitrogen Cat No NP0321BOX).

10 µl of sample (pooled fractions from IEX, or from affinity columns) was mixed with 5ul NuPAGE® LDS Sample Buffer (4X) (Invitrogen Cat No: NP0007), 2 µl

NuPAGE® Reducing Agent (10X) (Invitrogen Cat No: NP0004) and 3 µl deionized Water. The samples were heated to 95°C for 5 minutes. The running buffer was prepared by adding 50 ml 20X NuPAGE® MOPS SDS Running Buffer (Invitrogen Cat No: NP0001) to 950 ml of deionized water. The inner buffer chamber was filled with 200 ml 1X NuPAGE® SDS Running Buffer containing 500 µl NuPAGE® Antioxidant (Invitrogen Cat No: NP0005). The outer buffer chamber was filled with 600 ml 1X NuPAGE® SDS Running Buffer. 20 µl of sample was loaded onto the gel, which was subjected to 200 V over 1 hour. N.B if a Western was carried out using precast gels, NuPAGE® Transfer buffer (20X) (Invitrogen Cat No: NP0006) was used for the transfer.

*Linear gels (non-commercial): Preparation of 7.5% polyacrylamide gel.*

*Resolving gel:* 10.24 ml deionised water (DW), 4.5 ml 40% Bis-acrylamide, 9 ml 1M Tris.HCl pH 8.8, 120µl SDS, 120 µl ammonium persulfate (10% solution), 16 µl TEMED were mixed and slowly fed into the gap between glass plates. Water saturated butanol was placed on top of the gel and the gel was given time to set (30-45 minutes). Once set, water saturated butanol was removed and the gel rinsed twice with DW.

*Stacking gel:* 7.42 ml DW, 1.25 ml 40% Bis-acrylamide, 1.25 ml 1M Tris.HCl pH 6.8, 50 µl SDS, 50 µl ammonium persulfate (10% solution), 10 µl TEMED were mixed and layered on top of the resolving gel. The lane comb was inserted and the gel allowed to set (30-45 minutes).

*Pre-run:* Once the stacking gel was set, the rubber insert (on the bottom of the plates) was removed. The gel was then placed in an electrophoresis running tank and running buffer was added (3 g Tris, 144g glycine, 1% SDS, made up to 1 litre in DW) to a level above the comb. The comb was removed and the wells rinsed thoroughly with running buffer.

*Sample preparation:* 0.5-5 µg of sample (in a volume of no more than 20 µl) was mixed with sample (Laemmli) buffer (2.4 ml 1M Tris pH 6.8, 0.8g SDS, 4 ml 100% glycerol, 0.01% bromophenol blue, 1 ml 2 M DTT (fresh), 2.8 ml water). The mixture was heated to 94°C for 5 minutes before the samples were loaded into the wells. Full range molecular markers for size analysis (Invitrogen) were run in one

well. The gels were run for approximately 1 hour (until sample buffer reached base of gel) at 100 V.

## **2.6 Silver staining of proteins on SDS polyacrylamide gels**

After electrophoresis the gel was fixed for 1-2 hours in 50 ml Methanol, 12 ml Acetic acid, 50  $\mu$ l formaldehyde (37%) and 38 ml DW on a shaker at RT. The fixed solution was removed and the first equilibrating solution was added (50 ml Ethanol, 50 ml DW) for 10 minutes on a shaker at RT. The first equilibrating solution was removed and replaced by the second (30 ml ethanol, 70 ml DW) for 10 minutes on a shaker at RT. All other solutions were now prepared in advance as oxidation and developing is rapid. The proteins were oxidised by manually shaking the gel for 1 min with 20 ml  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  (1 mg/ml) plus 80 ml DW. The gel was washed three times in 100 ml DW, 20 seconds per wash. Silver nitrate solution (100 ml 0.1%  $\text{AgNO}_3$ , 75  $\mu$ l formaldehyde 37%) was added and the gel was placed on a shaker at RT for 20 minutes. The gel was then washed twice for 20 seconds in 100 ml DW. The developer was added (100 ml 6%  $\text{Na}_2\text{CO}_3$ , 50  $\mu$ l formaldehyde [37%], 40  $\mu$ l  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  [1 mg/ml]) and the gel was shaken manually until the  $\text{Ag}_2(\text{CO}_3)_2$  was seen to stain the gel. The reaction was stopped by adding 5% acetic acid to the gel for 5 minutes on the shaker at RT. Finally the gel was washed for 10 minutes in DW before being dried on blotting paper using a heated vacuum pump.

## **2.7 Alcian Blue staining of sialylated glycoproteins on SDS polyacrylamide gels**

After electrophoresis the gel was fixed for 1-2 hours in 50 ml methanol, 1 ml acetic acid, 49 ml DW on a shaker at RT. The fixative was removed and the gel equilibrated with 25 ml ethanol, 10 ml acetic acid, 65ml DW twice for 15 minutes on a shaker at RT. The gel was stained with 100ml of Alcain blue solution (0.125% Alcian Blue in 25% ethanol, 10% acetic acid in DW) for 20-30 minutes on a shaker at RT. The gel



was destained with 50% ethanol, 10% acetic acid in DW for three sets of 10 minutes, or until satisfied with staining contrast ratio. If excessive gel shrinkage occurred, the gel was rehydrated with 1% acetic acid in DW for 30 minutes. Finally the gel was dried on blotting paper using a heated vacuum pump.

## **2.8 Western blot**

*Gel electrophoresis was performed as described.*

*Transferring the protein to the membrane:* The gel was soaked in transfer buffer (running buffer + 20% methanol) for 5 minutes along with 6 pieces of appropriately sized blotting paper and a piece of hybond transfer membrane (Amersham Biosciences). A sandwich of the above was created - 3 pieces of blotting paper, the membrane, the gel and three pieces of blotting paper – and placed in a cartridge in a transfer tank. Transfer buffer was added to the tank until the cartridge was covered. The transfer takes approximately 1 hour 20 minutes at 100 V, or can be left overnight at 4°C at 30 V (both 50 mA).

*Antibody staining of the bound proteins:* The membrane was rinsed in Ponceau S for 5 minutes, to check for successful protein transfer, before being rinsed in PBS. The membrane was blocked in 2% BSA, 0.2% Tween 20 and 0.05% NaN<sub>3</sub>, in PBS for >2h on a rocker at RT. The membrane was rinsed in PBS before the primary antibody or biotinylated lectin was added (at 1 µg/ml, or 1:1000) in 1% BSA, 0.1% Tween 20 in PBS on a rocker at RT for >1 hour. The membrane was then rinsed three times over an hour with PBS + 0.02% Tween 20. The secondary antibody was then added (1:1000 or 1 µg/ml) e.g. anti-mouse IgG HRP or streptavidin HRP 1% BSA, 0.1% Tween 20 in PBS on a rocker at RT for >1 hour. The membrane was then rinsed twice with PBS + 0.02% Tween 20 and once with PBS over an hour.

*Developing:* The blot was developed using the ECL method following the manufacturer's instructions. A 1:1 mix of the ECL solutions was applied to the membrane and the resultant luminescence was developed using photosensitive film.

## **2.9 *IgG2aFc ELISA from purified fraction***

20 µl (plus 80 µl PBS) from every fourth elution fraction was plated on a 96 well ELISA plate (IWAKI) in duplicate and incubated overnight at RT. Standard: Mouse IgG1 control (R and D cat No MAB002) 1 in 2 dilutions from 5 µg/ml in duplicate. The wells were washed three times with 200 µl 0.05% Tween 20 in PBS before being blocked with 1% BSA in PBS for 2 hours at RT. The wells were washed three times with 200 µl 0.05% Tween 20 in PBS before 100 µl of a secondary (anti-mouse IgG-HRP) was added at a 1:500 dilution for 2 hours at RT. The plate was washed three times (0.05% Tween in PBS) before the substrate (Substrate reagent, R and D systems DY999; a 1:1 ration of H<sub>2</sub>O<sub>2</sub> and tetramethylbenzidine) was added and the plate was incubated for 20 minutes in the dark at RT. 50 µl stopping solution (1 M H<sub>2</sub>SO<sub>4</sub>) was then added to each well and the plate was read on a microplate reader at 450 nm. Concentrations of IgG2aFc in each fraction were calculated against the standard curve.

## **2.10 *MUC1 Ab ELISA on recombinant MUC1 glycoforms***

ELISA plates (IWAKI) were coated with 500 ng (100µl) of MUC1-IgG glycoform and incubated overnight at RT. The wells were washed three times with 200 µl 0.05% Tween 20 in PBS before being blocked with 1% BSA in PBS for 2 hours at RT. The wells were washed three times with 200 µl 0.05% Tween 20 in PBS before biotinylated MUC1-specific mAbs were added in serial 2-fold dilutions from 20 µg/ml to 2 ng/ml. Following incubation the wells were washed three times with 200 µl 0.05% Tween 20 in PBS before adding the substrate composed of peroxidase-conjugated streptavidin (DakoCytomation) for 30 minutes. After further washing with PBS the substrate *O*-phenylene-diamine (Sigma-Aldrich.) was added for 10 minutes at RT. Absorbance (OD 450) was measured using a Dynex MRX II (Jencons).

## **2.11 HMFG2/HMFG2 sandwich ELISA**

100 µl HMFG2 at a concentration of 1 µg/ml in PBS was plated on a 96 well ELISA plate (IWAKI) and incubated overnight at RT. The wells were washed three times with 200 µl 0.05% Tween 20 in PBS before being blocked with 1% BSA in PBS for 2 hours at RT. The wells were washed three times with 200 µl 0.05% Tween 20 in PBS before 100 µl standards (2 µg/ml of the MUC1 glycoform being assessed, in PBS, with serial 1 in 2 dilutions in duplicate) and 100 µl samples added in duplicate and incubated for 2 hours at RT. NB all samples and controls diluted in PBS. 100 µl biotinylated HMFG2 was added at a concentration of 1 µg/ml in PBS and incubated for 2 hours at RT. The wells were then washed three times with 200 µl 0.05% Tween 20 in PBS before streptavidin-HRP is added (1:200) in PBS and incubated for 20 minutes at RT in the dark. The wells were then washed twice with 200 µl 0.05% Tween 20 in PBS before a final wash in PBS alone. The substrate solution (Substrate reagent, R and D systems DY999; a 1:1 ratio of H<sub>2</sub>O<sub>2</sub> and tetramethylbenzidine) was added and the plate was incubated for 20 minutes in the dark at RT. 50 µl stopping solution (1 M H<sub>2</sub>SO<sub>4</sub>) was then added to each well and the plate was read on a microplate reader at 450 nm. Concentrations of MUC1 were calculated against the standard curve.

## **2.12 5E5/5E5 sandwich ELISA**

100 µl 5E5 at a concentration of 1 µg/ml in PBS was plated on a 96 well ELISA plate (IWAKI) and incubated overnight at RT. The wells were washed three times with 200 µl 0.05% Tween 20 in PBS before being blocked with 1% BSA in PBS for 2h at RT. The wells were washed three times with 200 µl 0.05% Tween 20 in PBS before 100 µl standards (2 µg/ml of the MUC1 glycoform being assessed, in PBS, with serial 1 in 2 dilutions in duplicate) and 100 µl samples added in duplicate and incubated for 2 hours at RT. NB all samples and controls diluted in PBS. 100 µl biotinylated 5E5 was added at a concentration of 1 µg/ml in PBS and incubated for 2

hours at RT. The wells were then washed three times with 200 µl 0.05% Tween 20 in PBS before streptavidin-HRP is added (1:200) in PBS and incubated for 20 minutes at RT in the dark. The wells were then washed twice with 200 µl 0.05% Tween 20 in PBS before a final wash in PBS alone. The substrate solution (Substrate reagent, R and D systems DY999; a 1:1 ration of H<sub>2</sub>O<sub>2</sub> and tetramethylbenzidine) was added and the plate was incubated for 20 minutes in the dark at RT. 50 µl stopping solution (1 M H<sub>2</sub>SO<sub>4</sub>) was then added to each well and the plate was read on a microplate reader at 450 nm. Concentrations of MUC1 were calculated against the standard curve.

### **2.13 The LAL endotoxin assay**

The Endpoint Chromogenic Limulus Amebocyte Lysate (LAL) Test is a quantitative test for Gram-negative bacterial endotoxin. It utilises the horseshoe crab's normal physiology. Instead of a mammalian clotting cascade after exposure to oxygen or collagen, upon contact with endotoxin (ubiquitous in the crab's environment), the crab's amebocytes trigger coagulation.

Samples were mixed with the LAL supplied in the test kit (Lonza, LAL QCL-1000, Cat No 50-647U) and incubated at 37°C (±1°C) for 10 minutes. A substrate solution was then mixed with the LAL-sample and incubated at 37°C (±1°C) for an additional 6 minutes. The reaction was stopped with the stop reagent supplied. The absorbance of the sample was determined spectrophotometrically at 405-410 nm. Since this absorbance is in direct proportion to the amount of endotoxin present, the concentration of endotoxin can be calculated from a standard curve.

### **2.14 Determining concentration by protein hydrolysis for amino acid content**

When the composition of an analysed protein is known, it is possible to exploit the fact that some amino acids such as: aspartate-asparagine, glutamate-glutamine,

alanine, leucine, phenylalanine, lysine and arginine are recovered well after hydrolysis. The concentration in n.mole/ml resulting from analysis can be converted to weight/ml present in the sample, and as % composition for each amino acids of a known protein is available, sample concentration can be determined.

e.g.

<i>Amino acid</i>	<i>n.mole/ml</i>	<i>µg/ml</i>	<i>% by frequency in recombinant MUC1</i>	<i>Total protein (µg/ml) in sample</i>
Alanine	112.000	7.96	17.3	46.01

## 2.15 Batches of MUC1 glycoforms used in Chapter 4

<b><i>Purified By:</i></b>	<b><i>Code</i></b>	<b><i>Glycan</i></b>	<b><i>Amount in mg</i></b>	<b><i>Endotoxin level EU/ml</i></b>	<b><i>EK cleaved</i></b>
<i>Gianfranco Picco</i>	<i>GP001</i>	<i>ST</i>	<i>2.5</i>	<i>&lt;5</i>	<i>Yes</i>
<i>Gianfranco Picco</i>	<i>GP001</i>	<i>T</i>	<i>1</i>	<i>&lt;5</i>	<i>Yes</i>
<i>Gianfranco Picco</i>	<i>GP002</i>	<i>ST</i>	<i>3</i>	<i>&lt;5</i>	<i>Yes</i>
<i>Gianfranco Picco</i>	<i>GP002</i>	<i>T</i>	<i>1</i>	<i>&lt;5</i>	<i>Yes</i>
<i>Gianfranco Picco</i>	<i>GP004</i>	<i>No glycans</i>	<i>4</i>	<i>&lt;5</i>	<i>Yes</i>
<i>Gianfranco Picco</i>	<i>GP005</i>	<i>Tn</i>	<i>3.5</i>	<i>&lt;5</i>	<i>Yes</i>
<i>Gianfranco Picco</i>	<i>GP014</i>	<i>STn</i>	<i>0.2</i>	<i>&lt;5</i>	<i>Yes</i>
<i>EU consortium (Partner 3 see appendix II)</i>	<i>PH5202</i>	<i>ST</i>	<i>3</i>	<i>&lt;5</i>	<i>No</i>
<i>EU consortium (Partner 3 see appendix II)</i>	<i>PH5258</i>	<i>T</i>	<i>1</i>	<i>&lt;5</i>	<i>No</i>
<i>EU consortium (Partner 3 see appendix II)</i>	<i>PH6162</i>	<i>ST</i>	<i>4</i>	<i>&lt;5</i>	<i>Yes</i>
<i>EU consortium (Partner 3 see appendix II)</i>	<i>PH6289</i>	<i>T</i>	<i>1.5</i>	<i>&lt;5</i>	<i>Yes</i>

## ***Materials and methods used in chapter 4***

### ***2.16 Immunization with recombinant MUC1 glycoproteins***

All animal work was carried out under Project Licence Number: PPL 70/6847, strictly adhering to Home Office guidelines

Lyophilised recombinant MUC1 glycoproteins were reconstituted in endotoxin free PBS at a concentration of 400 µg/ml, prior to been mixed with an equal volume of Freund's adjuvant to form a water in oil emulsion. Initial injections were composed of an equal volume of reconstituted MUC1 glycoprotein and Freund's complete adjuvant (Sigma F5881), and were administered in a total volume of 100 µl subcutaneously into right flank of mice. Subsequent immunizations were administered at three weekly intervals with reconstituted MUC1 glycoproteins mixed with Freund's incomplete adjuvant (Sigma 5506), and a total volume of 100 µl administered subcutaneously into alternate flanks of mice. Seven days following the last immunization, mice were sacrificed and the spleen removed for *in vitro* testing.

### ***2.17 Adjuvant in vivo treatment***

Freund's was chosen as adjuvant as it is the most commonly used adjuvant in research. It is water in oil emulsion composed of 85% paraffin oil and 15% mannide monooleate (Freund's incomplete, Sigma F 5506), or additionally containing 1 mg/ml of heat-killed and dried *Mycobacterium tuberculosis* (strain H37Ra, Freund's complete, Sigma F5881). Freund's adjuvant was first developed by Jules Freund in the 1940's, and designed to provide continuous release of antigens necessary for stimulating a strong, persistent immune response (Freund J. and McDermott K. 1942). Antigens tested as immunogen in PBS were mixed with an equal volume of the Freund's adjuvant to form an emulsion, and immunizations were given subcutaneously in a total volume of 100 µl. The mycobacterium in Freund's complete has been found to attract macrophages and other cells to the injection site,

which enhances the immune response. For this reason, Freund's complete was used for the initial injections. Because Freund's complete causes granulomas inflammation at the inoculation site, in accordance with UK guidelines, Freud's incomplete was used for the following boosts.

## **2.18 *T cell assay***

For splenocyte extraction, mouse spleens were squashed and suspended in 35 ml RPMI followed by centrifugation (200 G for 5 min) and resuspended in 10 ml RPMI. 10 ml Ficoll-Paque<sup>TM</sup> PLUS (GE Healthcare) was then layered beneath. After centrifugation (400 G 15 min with brake turned off) the splenocytes were transferred to a new tube and an equal volume of RPMI was added followed by centrifugation (200 G for 10 min). Cells were washed twice in RPMI and resuspended in 4-8 ml RPMI containing 5% FCS at  $10^7$  cells/ml. Finally, cells were filtered through a 50  $\mu$ M filter to eliminate clumps. For stimulation of splenocytes,  $1 \times 10^6$  cells in 100  $\mu$ l culture medium were incubated with 1  $\mu$ g of the recombinant glycoprotein used as immunogen, or control (non specific glycoprotein) for 48-60 hours at 37°C and 5%CO<sub>2</sub>. As a positive control cells were incubated with 1  $\mu$ g Concanavalin A lectin. Cytokine concentrations (IFN-commercially available two-site sandwich enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (R&D Systems). Experiments were performed in triplicate and supernatants were collected after 48 hours of CTL induction.

## **2.19 *Bone marrow derived DC preparation***

Mouse femur bones (2 bones from 1 mouse) were dissected above and below the joint (knee and hip), and using small dissection scissors the majority of muscles were removed prior its transfer into a dish containing RPMI medium. Bones were then held with tweezers, and using scissors, both end of the bone were cut to expose the bone marrow. The marrow was washed out with RPMI media using a syringe. Gentle pipetting up and down of the media containing the bone marrow using a syringe was

performed in order to prepare a single cell suspension, prior to plating the cells in 10 cm culture dish at a concentration of a million cells per ml in IMDM media containing 5% Foetal Calf Serum (FCS), 20 ng/ml GM-CSF and 200 IU/ml IL-4. On day 3 of cell culture (37°C with 10%CO<sub>2</sub>), media was removed and a gentle wash with fresh media was applied using a pipette to remove non-adherent cells from the culture. The adherent cells were then supplied with fresh media and placed back into culture. On day 6, culture media was removed and non-adherent DC cells were separated from adherent progenitor by gently washing the culture dish, held at 45 degree angle, with PBS using a pipette. The DCs were collected to be used for further testing.

## **2.20 *In vitro* T-cell depletion**

*In vitro* CD4 and/or CD-8 T cells subsets were separated from mouse single cell suspensions using MACS<sup>®</sup> technology from Miltenyi Biotec. Cells in a single cell suspension were labelled with CD4 (L3T4) bound to magnetic microbeads (catalogue number 130-049-201), or CD8 (Ly-2) microbeads (catalogue number 130-049-401). Samples were then applied to MACS column in a MACS separator, which retains the labelled cells. The flow-through was collected as the unlabelled cell fraction. Cells positively selected for CD4 or CD-8 T cells subsets were then used for *in vitro* testing.

## **2.21 *In vivo* T-cell depletion**

*In vivo* depletion of CD4 and/or CD-8 T cells subset was performed using rat anti-CD4 (Clone GK1.5) and rat anti-CD8 (Clone 2.43) MAb. The Abs were produced by Cancer Research UK from hybridoma cells and shown to be endotoxin free. Initial depletion in adult mice was performed by injecting the antibodies intraperitoneally for three consecutive days, at a concentration of 20 mg/kg diluted into 100 µl of endotoxin free PBS. To determine the depletion efficiency at day 6 mice were sacrificed, and a single cell suspension from the spleens was made. Flow cytometry



was performed on the cells after staining with directly labelled (FITC) anti-CD4 or anti-CD8 MAbs. Maintenance of the depletion was obtained by injecting 20 mg/kg the antibodies intraperitoneally every three days.

## **2.22 Flow cytometric analysis**

Cells were gently removed from culture, counted using a CASY counter, and spun down at 200 G before being resuspended at  $5 \times 10^5$  cells/ml in cold 0.5% BSA in PBS. 200  $\mu$ l ( $1 \times 10^5$  cells) of this solution per test was placed in a round bottom 96 well plate and incubated in buffer for 10 minutes to block non-specific binding before being spun down at 200 G and resuspended in 50  $\mu$ l cold 0.5% BSA in PBS. Appropriate amount of each antibody was then added to the appropriate well and the cells were incubated for 45 minutes on ice in the dark. Cells were then washed in 200  $\mu$ l/well cold 0.5% BSA in PBS and resuspended in 150  $\mu$ l cold 0.5% BSA in PBS and 100  $\mu$ l 1% paraformaldehyde solution (in PBS). The cells were then run through the flow cytometer (Beckman Coulter Epics XL) and analysed using winMDI software.

## **2.23 IFN $\gamma$ sandwich ELISA (R & D; DY285)**

100  $\mu$ l of 4  $\mu$ g/ml capture anti-mouse IFN $\gamma$  antibody in PBS was plated on a 96 well ELISA plate (IWAKI) and incubated overnight at RT. The wells were washed three times with 200  $\mu$ l 0.05% Tween 20 in PBS before being blocked with 1% BSA in PBS for 2 hours at RT. The wells were washed three times with 200  $\mu$ l 0.05% Tween 20 in PBS before 100  $\mu$ l standards (serial 1 in 2 dilutions from 2000 pg/ml) and 100  $\mu$ l samples added in duplicate and incubated for 2 hours at RT. All samples and controls were diluted in reagent dilutant: 1% BSA in PBS. 100  $\mu$ l biotinylated anti-mouse IFN $\gamma$  was added at a concentration of 50 ng/ml in reagent dilutant and incubated for 2 hours at RT. The wells were then washed three times with 200  $\mu$ l 0.05% Tween 20 in PBS before streptavidin-HRP was added (1:200) in reagent

dilutant and incubated for 20 minutes at RT in the dark. The wells were then washed twice with 200  $\mu$ l 0.05% Tween 20 in PBS before a final wash in PBS alone. The substrate solution (Substrate reagent, R and D systems DY999; a 1:1 ratio of H<sub>2</sub>O<sub>2</sub> and tetramethylbenzidine) was added and the plate was incubated for 20 minutes in the dark at RT. 50  $\mu$ l stopping solution (1M H<sub>2</sub>SO<sub>4</sub>) was then added to each well and the plate was read on a microplate reader at 450nm. Concentrations of IFN $\gamma$  were calculated against the standard curve.

## ***2.24 Detection of antibodies from mouse serum by ELISA***

100  $\mu$ l of PBS containing 500 ng of either recombinant MUC1, 60mer MUC1 peptide or lipopolysaccharides LPS were placed on each well of a 96 well ELISA plate (IWAKI) and incubated overnight at room temperature (RT). The wells were washed three times with 200  $\mu$ l 0.05% Tween 20 in PBS before being blocked with 1% BSA in PBS for 2 hours at RT. The wells were washed three times with 200  $\mu$ l 0.05% Tween 20 in PBS before 100  $\mu$ l of mouse serum diluted 1/40 in PBS (prior and after immunizations) was applied in duplicate to the ELISA's plate wells and incubated for 2 hours at RT. The wells were washed three times with 200  $\mu$ l 0.05% Tween 20 in PBS before 100  $\mu$ l of a secondary (anti-mouse IgG-HRP) was added at a 1:500 dilution for 2 hours at RT. The plate was washed three times (0.05% Tween in PBS) before the substrate (Substrate reagent, R and D systems DY999; a 1:1 ratio of H<sub>2</sub>O<sub>2</sub> and tetramethylbenzidine) was added and the plate was incubated for 10 minutes in the dark at RT. 50  $\mu$ l stopping solution (1 M H<sub>2</sub>SO<sub>4</sub>) was then added to each well and the plate was read on a microplate reader at 450 nm.

## ***2.25 Biotinylation of antibody and recombinant MUC1 glycoforms***

The biotinylation buffer was made (51.9 g NaHCO<sub>3</sub> and 25.8 g Na<sub>2</sub>CO<sub>3</sub> in 3 litres DW pH 9.30) and chilled to 4°C before use. A minimum of 0.5 mg/ml solution of

Abs or MUC1-glycoforms were made up and placed in dialysis tubing (NB for unglycosylated MUC1 biologically inert, biotech cellulose ester is used to minimise loss; Spectrumlabs ‘float-a-lyzer’ G2; G235031) before being placed in the buffer at 4°C overnight. The buffered Abs or MUC1 glycoforms were then mixed with 120 µg (per mg) biotin/DMSO premade stocks (1 mg/ml). The mix was rotated at RT for a minimum of 4 hours before being dialysed against cold 3 litres PBS + 0.1% azide overnight at 4°C. The Abs or MUC1 glycoforms were removed from the dialysis tubing and the concentration was calculated from the change in volume.

## ***Materials and methods used in chapter 5***

### ***2.26 Development of hST3Gal-I transgenic mice***

Human ST3Gal-I (+889 to +1951) (Accession number: L29555, EC:2.4.99.4) was amplified by PCR from a plasmid containing hST3Gal-I full cDNA with the following primers designed to insert restriction sites for Not I at both 5’ and 3’ ends of the amplicon:

Forward primer: 5’-ATCAGCTAGCGCGCCGCTTCCTGCTACCCATCGT-3’

Reverse primer: 5’-TAGAAGTTCCCCTCTACTCCTAGGCGC-3’.

Using these Not I sites, the resulting cDNA was inserted into the pNASSβ plasmid (Clontech), downstream of the promoter region of human MUC1 (from -1401 to +33) previously cloned in this plasmid (Graham R. A. *et al.* 2001). A 2.5kb Sal I-Sca I fragment containing the MUC1 promoter fused to ST3Gal-I was purified (Qiagen kit) and dissolved in injection buffer (10 mM Tris, 0.1 mM EDTA pH 7.4 prepared in ultrapure water). DNA (5 ng/l) was injected into the pronucleus of pure C57/Bl6 mouse embryos that were subsequently transferred into day 1-plugged pseudopregnant C57/Bl6 female mice (Injections performed by CR-UK Biological Resource unit).

Transgenic mice were identified by PCR on DNA isolated from tail snips, amplifying a 550 bp segment of DNA using oligonucleotides primers within the 3' end of the MUC1 promoter and within the 5' end of the ST3Gal-I:

Forward primer: 5'-GCCGTTGTGGGCAACTCGGGC-3'

Reverse primer: 5'-GCTCACCACCCACTCCAAGTCT-3'.

Two founder mice were identified that expressed hST3Gal-I in the expected tissues and from one founder, mice homozygous for hST3Gal-I were developed by littermate mating. Homozygosity was confirmed by backcrossing mice onto WT C57Bl/6, which resulted in 100% of the offspring carrying hST3Gal-I.

## ***2.27 Development of spontaneous tumours in hST3Gal-I transgenic mice***

Homozygous hST3Gal-I female mice on a pure C57Bl/6 background were mated with FVB male mice heterozygous for the Polyomavirus middle T antigen driven by the mouse mammary tumour virus (MMTV-PyMT mice (Guy C. T. *et al.* 1992) promoter to obtain the ST3Gal-I/PyMT mice. Heterozygous males mice for the Polyomavirus middle T antigen were used for breeding as spontaneous tumours appear at a very early age on homozygous and also heterozygous MMTV-PyMT mice female mice. Control mice (Control/PyMT mice) were derived by crossing female C57Bl/6 mice with MMTV-PyMT male mice. The offspring were screened for the polyoma middle T antigen by PCR of DNA prepared from tail snips using the following primers:

Forward primer: 5'-CCAGAACTCCTGTATCCAGAAGCG-3'

Reverse primer 5'-GGATGAGCTGGGGTACTTGTTCCTCC-3'.

Female mice carrying the PyMT were examined three times per week for the development of tumours.

## 2.28 Immunohistochemistry

Frozen sections of mouse tissue were fixed in ice-cold acetone for 10 minutes, and formalin fixed paraffin embedded sections were used after xylene removal of paraffin and gradual ethanol rehydration. For staining for mouse Muc1, endogenous peroxidase activity was blocked in 1/100 H<sub>2</sub>O<sub>2</sub>/methanol for 10 minutes. After rehydration and/or blockage of endogenous peroxidase activity, sections were blocked with 50% foetal calf serum and then incubated with a monoclonal antibody 4B10 to human ST3Gal-I (Vallejo-Ruiz V. *et al.* 2001) or a polyclonal antibody to the human MUC1 cytoplasmic tail that also reacts with mouse Muc1 (Pemberton L. *et al.* 1992). After extensive washing the slides were incubated with biotinylated rabbit anti-mouse Ig or biotinylated goat anti-rabbit Ig (DAKO), respectively, for 30 minutes, washed and incubated with streptavidin biotin complexes linked to horse radish peroxidase (DAKO). Binding was visualised with diaminobenzidine (DAKO) and the sections counter-stained with haematoxylin. Haematoxylin and eosin (H&E) staining on formalin-fixed paraffin embedded tissue was used for additional histological analysis.

## 2.29 Glycosyltransferase assays *(performed by Prof. Inka Broakhausen's group)*

Homogenates of mouse tissues were prepared by mincing tissues and hand homogenizing in 5 times volume of 0.25 M sucrose. Protein contents were determined by the BioRad protein assay (Bradford) using BSA as the standard. All enzymes were assayed in at least duplicate determinations, and results varied by <10% between duplicates. Assays lacking exogenously added acceptor substrate provided the background radioactivity.

### *Sialyltransferase*

ST3Gal transferase assay mixtures contained in a total volume of 40 µl: 2 mM Galβ1-3GalNAcα-p-nitrophenyl (Galβ1-3GalNAcα-pnp) acceptor substrate, 0.1 M

Tris-HCl, pH 7, 0.125 % Triton X-100, 5 mM MnCl<sub>2</sub>, 0.4 mM CMP-[<sup>3</sup>H]sialic acid (2420 cpm/nmol), and 10 ml homogenate (0.08 to 0.15 mg protein). The mixtures were incubated for 1 hour at 37°C. Reactions were stopped with 0.2 ml water and freezing, and mixtures filtered through 0.1 ml of Bio-Gel P2, washed three times with 0.4 ml water. After lyophilizing combined eluates, 120 ml water were added and 80 ml injected into HPLC, using an analytical amine column and acetonitrile/15 mM K-phosphate, pH 5.2 = 75/25 as the mobile phase. The absorbance at 195 nm and radioactivity of 2 minutes fractions were monitored. Sialic acid eluted at about 25-30 min and enzyme product at 14-18 min. Radioactivity of fractions was determined by scintillation counting. Radioactive CMP-sialic acid did not elute under these conditions, but can be eluted with acetonitrile/15 mM K-phosphate, pH 5.2 = 50/50.

#### *Polypeptide GalNAc-transferase*

Polypeptide GalNAc-transferase activity was assayed in mixtures containing in a total volume of 40 ml: 0.5 mM acceptor substrate AQTPPP, 0.125 M MES buffer, pH 7, 0.125 % Triton X-100, 10 mM AMP, 10 mM MnCl<sub>2</sub>, 0.9 mM UDP-[<sup>3</sup>H]GalNAc (3795 cpm/nmol), and 10 ml homogenate (0.08 to 0.15 mg protein). The mixtures were incubated for 1 hour at 37°C. Reactions were stopped with 0.6 ml water and freezing. Mixtures were applied to columns of 0.4 ml AG1x8, which were washed twice with 0.6 ml water. Eluates were lyophilized and enzyme product separated by HPLC using a C18 column and acetonitrile/water = 6/94 as the mobile phase. The absorbance at 195 nm and radioactivity of 2 minutes fractions were monitored. Radioactivity of fractions was determined by scintillation counting.

#### *Core 2 β6GlcNAc-transferase*

Core 2 β6GlcNAc-transferase assay mixtures contained in a total volume of 40 ml:

2 mM Galβ1-3GalNAcα-Bn acceptor substrate, 0.125 M GlcNAc, 0.125 M MES buffer, pH 7, 0.125 % Triton X-100, 10 mM AMP, 0.5 mM UDP-[<sup>3</sup>H]GlcNAc (5230 cpm/nmol), and 10 ml homogenate (0.06 to 0.24 mg protein). The mixtures were incubated, in duplicates, for 1 hour at 37°C. Product was isolated as described for

polypeptide GalNAc-transferase by AG1x8 and HPLC using a C18 column and acetonitrile/water = 12/88 as the mobile phase.

### **2.30 Analysis of O-glycans of mice tumours** (performed by Prof. Anne Dell's group)

Snapfrozen mouse tumours were sent to Imperial College on dry ice where they were processed. All mouse tumours were treated as described in (Sutton-Smith M. *et al.* 2007). Briefly, each tumour sample was subjected to reduction in 4M guanidine-HCl (Pierce, Cramlington, Northumberland, UK), carboxymethylation and trypsin digestion. The digested glycoproteins were purified by C<sub>18</sub>-Sep-Pak (Waters Corp, Hertfordshire, UK). O-glycans were released by reductive elimination and then permethylated using the sodium hydroxide procedure. Finally, the permethylated O-glycans were purified by C<sub>18</sub>-Sep-Pak.

MS and MS/MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems, Darmstadt, Germany) mass spectrometer. Permethylated samples were dissolved in 10 µl of methanol and 1 µl of dissolved sample was premixed with 1 µL of matrix (20 mg/ml 2,5-dihydroxybenzoic acid (DHB) in 70% (v/v) aqueous methanol), spotted onto a target plate (2×0.5 µl) and dried under vacuum. The collision energy was set to 1 kV, and argon was used as collision gas. The 4700 Calibration standard kit, calmix (Applied Biosystems), was used as the external calibrant for the MS mode and [Glu1] fibrinopeptide B human (Sigma-Aldrich) was used as an external calibrant for the MS/MS mode.

The MS and MS/MS data were processed using Data Explorer 4.9 Software (Applied Biosystems). The spectra were subjected to manual assignment and annotation with the aid of the glycobioinformatics tool GlycoWorkBench (Ceroni A. *et al.* 2008). The proposed assignments for the selected peaks were based on <sup>12</sup>C isotopic composition together with knowledge of the biosynthetic pathways. The proposed structures were then confirmed by data obtained from MS/MS experiments.

### **2.31 *TGF $\beta$ ELISA***

The concentration of TGF $\beta$  in mouse serum was measured by a commercially available two-site sandwich enzyme-linked immuno-absorbent assay (ELISA) from R&D Systems according to manufacturer's instructions. Assays were performed in duplicate and experiments repeated twice.

### **2.32 *Immunoprecipitation***

Snap-frozen tumours were crushed using a Mikro-dismembrator II (B Braun Biotech, Melsungen, Germany). Tumour powders were dissolved in lysis buffer (Tris/HCl 50 mM, NaCl 150 mM, Triton X100 0.1%) containing the complete-mini anti-protease cocktail (Roche, Lewes, UK) and incubated for 6 hours. Lysates were homogenised with an Ultra-Turrax T8 (Ika, Fisher Scientific, Loughborough, UK) and further incubated for 2 hours. Total tumour lysates (2 mg of proteins) were pre-cleared using 50  $\mu$ l of A/G proteins agarose-beads (Roche) for 2 hours. The lysates were then incubated with 5  $\mu$ g of anti-c-Src antibody (Santa Cruz) or 100  $\mu$ l of anti-CT supernatant (CT2 antibody), overnight at 4°C.

A/G proteins beads (100  $\mu$ l) were subsequently added and incubated for 4 hours. Immunoprecipitated proteins were washed using PBS, then eluted from the bead using elution buffer (0.1 M glycine at pH 2.5) for two incubations of 15 minutes each. All procedures were carried out at 4°C using ice-cold reagents.

### **2.33 *Electrophoresis and Western blotting***

The immuno-precipitates were loaded onto 4 to 12% gradient acrylamide gels, separated by SDS-PAGE electrophoresis under reducing conditions and electro-transferred on to nitrocellulose membranes (Biotrace NT; Gelman Science) in accordance with standard procedures. Membranes were blocked in 1% BSA in Tris



Buffered Saline (TBS) and incubated with primary antibodies, anti c-Src or a polyclonal antibody (CT1) to the cytoplasmic tail of Muc1, in TBS 0.05% Tween-20, for 1 hour. After washing, labelled proteins were revealed using appropriate secondary antibodies conjugated to alkaline-phosphatase and NBT/X-phosphate revelation reagent (Roche, Lewes, UK).

Western blots for AKT and Phospho-AKT were performed using antibodies from Cell Signaling Technology using a phospho-Ser473 specific antibody and visualized using ECL system (GE Healthcare).

### ***2.34 RNA extraction, reverse transcription and quantitative real-time-PCR***

Cell pellets were snap frozen in 50 µl of RNA Later reagent (Qiagen, Crawley, UK). Total RNA was extracted from cells using the Nucleospin RNA II kit (Macherey Nagel, Düren, Germany), according to manufacturer's instructions. The integrity of the extracted RNA was controlled using a Bioanalyser (Agilent Technologies, Wokingham, UK). DNased RNA (0.6 µg) was reverse transcribed using random hexamer primers and superscript III reverse transcriptase (Invitrogen, Paisley, UK). QRT-PCR was performed using SYBR Green I technology (Sigma-Aldrich, Poole, UK) and Opticon qRT-PCR analysis system (MJ Research, Waltham, MA). Briefly, each reaction mix contained 10 ng of cDNA and 10 pmol of the ST3Gal-I primers:

Forward primer: 5'-CGGTACCCGGGGATCAATTCGAG-3'

Reverse primer 5'-TCCCATCACAAATCACCATGGACC-3'.

Cycles were as follows: 15 seconds denaturation at 94°C, 30 seconds annealing at 60°C, 30 seconds extension at 72°C and fluorescence detection at 78°C, repeated 35 times.

# ***Chapter 3***

## ***Purification of recombinant MUC1 glycoproteins***

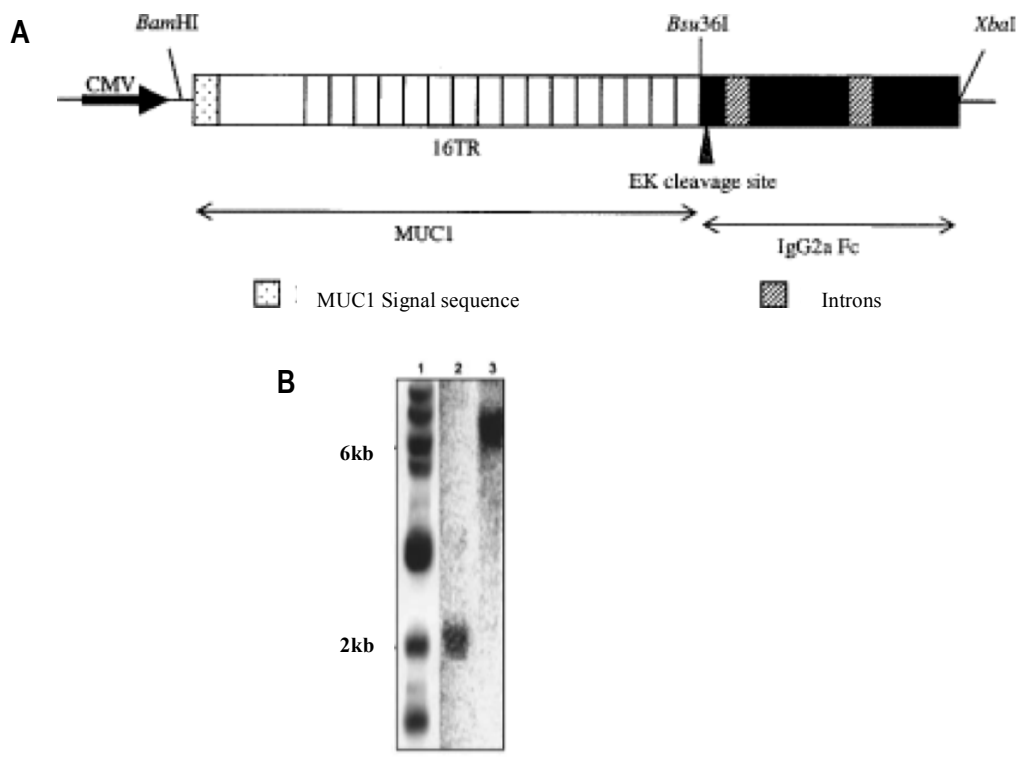
### **3.1 Introduction**

We hypothesized that aberrant glycoforms of MUC1 can induce an immune response and therefore have the potential to be used as immunotherapeutic agents. Therefore, the objective of this chapter was to purify specific tumour-associated glycoforms of MUC1 to be used as immunogens in experiments designed to evaluate their efficacy for the immunotherapy of breast and other MUC1 expressing cancers. In this chapter the purification of recombinant MUC1 glycoproteins expressing predominantly either the glycan Tn, Sialyl-Tn, T, Sialyl-T or no O-linked glycans will be described. These glycoforms were produced in CHO and CHO ldld cells, and purified from the media by anion exchange or affinity chromatography. Amino acids analysis, Western blotting, Silver staining and endotoxin testing confirmed the purity.

The utilization of mammalian cell lines for the production of biopharmaceutical agents has been studied since the late 1940s, and by the early 1980s biological agents produced in tissue culture included vaccines for polio, mumps and measles. Since then, a wide variety of cell lines have become available to produce biological agents, with the Chinese Hamster Ovary (CHO) cell line being the most widely used. CHO cells are well suited as a production cell line for many reasons, and these include the fact that the cells are widely available, fast growing, easily transfectable, can adapt well to growth in monolayer or in suspension and have been grown successfully in a variety of production systems. Furthermore, and most importantly in this case, MUC1 O-linked glycosylation carried out by CHO cells has been found to be very similar to that seen on MUC1 from breast cancer (Backstrom M. *et al.* 2003), represented as an extreme case by the cell line T47D (Lloyd K. O. *et al.* 1996) derived from a metastatic breast cancer. For these reasons CHO cells, either wild-type, mutant or expressing a specific glycosyltransferase were chosen for the large scale production of recombinant MUC1 glycoproteins expressing predominantly either the glycan Tn, Sialyl-Tn, T, Sialyl-T or with no O-linked glycans.

### **3.2 Large scale production of MUC1 glycoproteins**

As part of an EU funded consortium co-ordinated by the Breast Cancer Biology Group, recombinant MUC1, composed of the amino end of MUC1 and 16 tandem repeats fused to the Fc region of mouse IgG-2a (fig 3.1A) for efficient secretion, was expressed in CHO-K1 (Chinese-hamster ovary K1) cells, CHO ldlD mutants (Kingsley, D. M. *et al.* 1986) and CHO cells transfected with glycosyltransferases. The expression construct consisted of 16 tandem repeats, as shown by digesting the CHO DNA with the enzyme Hinf-1 that cuts just outside the tandem repeat region of MUC1 (fig 3.1B), and all of the sequence of MUC1 5' to the tandem repeats fused at the 3' end to exons 1-3 of the Fc region of mouse IgG2a. The cells were cultured in bioreactors, by the German partners in the EU consortium (see appendix II), and the supernatant was concentrated through ultrafiltration, mixed with a 50mM Tris/HCl pH8 buffer, and sent to us for further purification and testing. Additionally, the Swedish partner within the EU consortium determined the glycan profile of the purified MUC1 produced by CHO cells, which was shown to be mainly MUC1-ST, or MUC1-STn when the CHO cells were also stably transfected with the cDNA for the glycosyltransferase ST6GalNAc-I (Sewell R. *et al.* 2006) (table 3.1). Moreover, CHO ldlD cells, which are deficient for the enzyme UDP-galactose and UDP-N-acetylgalactosamine (GalNAc) 4-epimerase were transfected with the recombinant MUC1. This deficiency makes the cells incapable of converting glucose to galactose or N-acetylgalactosamine and therefore these cells cannot add any O-linked glycans. Thus, the use of these cells allows MUC1 without O-linked glycans to be purified. However, the defect can be reversed by providing the cells with exogenous sources of sugars (Kingsley, D. M. *et al.* 1986), so by culturing the cells in media containing GalNAc, MUC1 carrying only Tn can be produced.



**Figure 3.1** *MUC1 fused to the Fc region of mouse IgG2a used to express the mucin in CHO cells.*

*A: Schematic drawing of the MUC1-IgG construct used for the large-scale production in CHO-K1, and CHO ldlD mutant cells. B: Southern blot of restriction-enzyme-digested genomic DNA probed with a MUC1 TR-specific DNA probe. Lane 1, Molecular-mass marker; lane 2, digestion with HinfI (predicted size for 16-TR MUC1 gene 2.4 Kb); lane 3, digestion with EcoRI (predicted size for 16-TR MUC1 gene 6.4 Kb).*

**Table 3.1:** *Structure of O-glycans on MUC1 expressed in CHO cells and CHO cells transfected with ST6GalNAc-I*

Detailed comparison of O-glycans on MUC1-IgG produced in wild-type CHO cells (CHO MUC1) and in CHO MUC1 cells transfected with the human glycosyltransferase ST6GalNAc-I (CHO MUC1 ST6GalNAc-I).

<i>O</i> -glycans	Relative amounts %	
	<i>CHO MUC1</i> <i>ST6GalNAc-I</i>	<i>CHO MUC1</i>
Galβ1-3GalNAc- (T)	0	2
Neu5Acα2-6GalNAc- (STn)	83	0
Galβ1-3(Neu5Acα2-6)GalNAc- (ST)	1	1
Neu5Acα2-3Galβ1-3GalNAc- (ST)	4	84
Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc (Disialyl T)	12	13

### **3.3 Results**

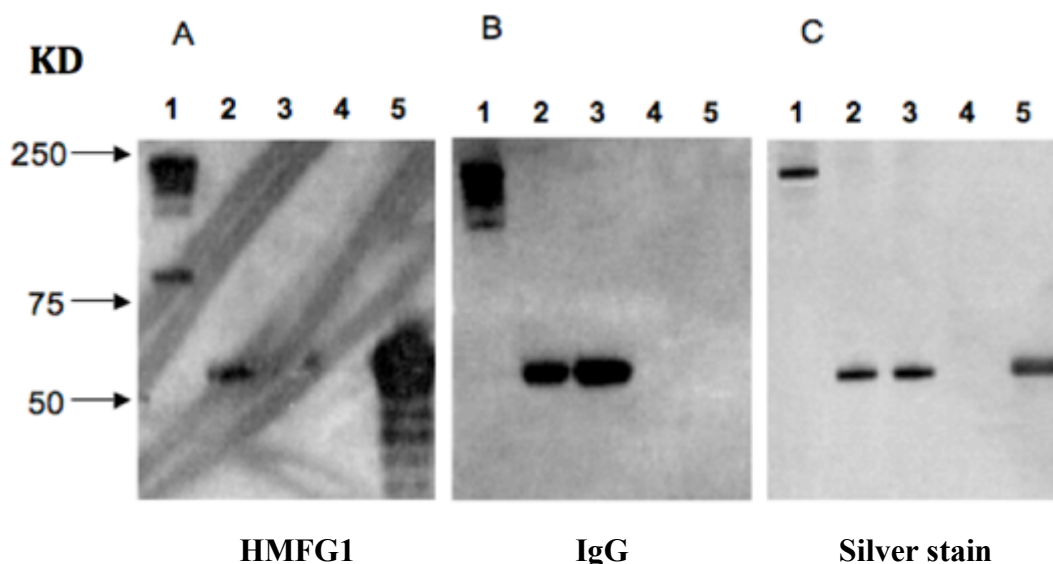
#### **3.3.1 Purification of MUC1 without O-linked glycosylation**

To purify MUC1 without O-linked glycosylation, MUC1-Ig transfected CHO 1d1D cells were cultured in the absence of GalNAc in bioreactors, concentrated and re-buffered as described in 3.2, before being sent to our lab. The IgG portion was cleaved off using 48 hours of enterokinase (EK) treatment (see materials and methods), and MUC1 without O-linked glycosylation purified by affinity chromatography, using the HMFG1 mAb (Taylor-Papadimitriou J. *et al.* 1981) bound to sepharose beads to capture the unglycosylated MUC1. Bound material was eluted from the column using 0.2 M glycine pH 2.5, and the MUC1 containing fractions identified by HMFG1 sandwich ELISA. Pooled fractions containing MUC1 were run on SDS PAGE followed by Silver staining and Western blotting (fig 3.2).

In figure 3.2A (HMFG1 Western blot) the majority of MUC1-IgG appears to migrate on a SDS PAGE gel at around 200 kDa (lane 1), and after EK cleavage a single band is notable at about 55 kDa level (lane 2) which is very close to the calculated size of 51 kDa for the unglycosylated MUC1-16TR. As no other bands are present in lane 2, all the starting material appears to be cleaved by the EK. Furthermore, the HMFG1 affinity column appears to retain all the MUC1, as no bands are present in the flow-through shown in lane 3 of the HMFG1 Western blot. Lane 5 shows the pooled fractions containing the eluted MUC1 with the major band being at 55 kDa. There appear to be a low level of degradation as a ladder appearance of bands at less than 50 kDa level is visible.

Figure 3.2B (anti-IgG Western blot) also indicates that the majority of MUC1-IgG appears to migrate on a SDS PAGE gel at around 200 kDa (lane 1), and confirms that the majority of the material is cleaved after treatment with EK, as a single band is present in lane 2. The Fc region of mouse IgG2a-Fc also, like MUC1-16TR, migrates on a SDS PAGE gel at around 55 kDa (lane 2), but is not retained by the HMFG1 column as a 55 kDa band is present in the flow-through material (lane 3), and absent from the eluted materials (lane 5).

To identify potential protein impurities a silver stain was performed (fig 3.2C). A single band was found in the starting material, after EK cleavage, in the flow-through and in the eluted fractions shown in lane 1, 2, 3 and 5 respectively. This indicates that the unglycosylated MUC1 had a good level of purity prior, during and after the removal of IgG.



**Figure 3.2** Purification of MUC1 without O-linked glycans

*Purification stages of MUC1 produced in CHO Id1D cells without the addition of exogenous sugars: Lane 1, MUC1-IgG; lane 2, after enterokinase cleavage treatment; lane 3, column flow-through; lane 4, empty; lane 5, pooled fractions 8-10 after elution with 0.2 M glycine pH 2.5.*

*A: HMFG1 Western blot. B: Anti-IgG Western blot. C: Silver staining.*

Purified unglycosylated MUC1 material was assessed by HMFG1 sandwich ELISA, using the quantified (by IgG ELISA) MUC1-IgG as a standard (fig 3.3A), to calculate the amount of purified material in the various eluted fractions. The total amount in the pooled fractions was found to be 0.5mg from each mg of starting material, and this agrees with the theoretical amount that can be purified, as both MUC1 and Fc portion have similar molecular weight (about 50 kDa). To further confirm the purity the unglycosylated MUC1 was sent for amino acid (a. a.) analysis. The purified product was extensively dialysed against PBS to remove the 0.2 M glycine used during the elution step, prior to being analysed. Unfortunately, possibly due to its stickiness, the majority of the unglycosylated MUC1 was lost during the dialysis step. However, enough was recovered for the a. a. analysis (performed by Alta Bioscience Birmingham), which showed a high level of concordance with the

theoretical composition. Only the amino acids glycine and glutamine and glutamic acid were substantially different from the theoretical level. In the case of glycine this was probably due to some glycine from the elution buffer still being present (table 3.2).

Figure 3.3B shows that HMFG1, HMFG2 and SM3 mAb (Taylor Papadimitriou J. *et al.* 1981, Burchell J. *et al.* 1989, Girling A. *et al.* 1989), three monoclonal antibodies to the core protein of MUC1, can bind to MUC1 purified from CHO Id1D cells but that the MUC1-Tn specific 5E5 (Sorensen A. L. *et al.* 2006) monoclonal antibody does not bind. This suggests that there was no GalNAc on the purified material. Moreover, as the migration pattern of the product on a SDS PAGE gel was found to be of the predicted size for unglycosylated MUC1 (about 51 kDa), it was concluded that the purified material contained no O-linked glycans.

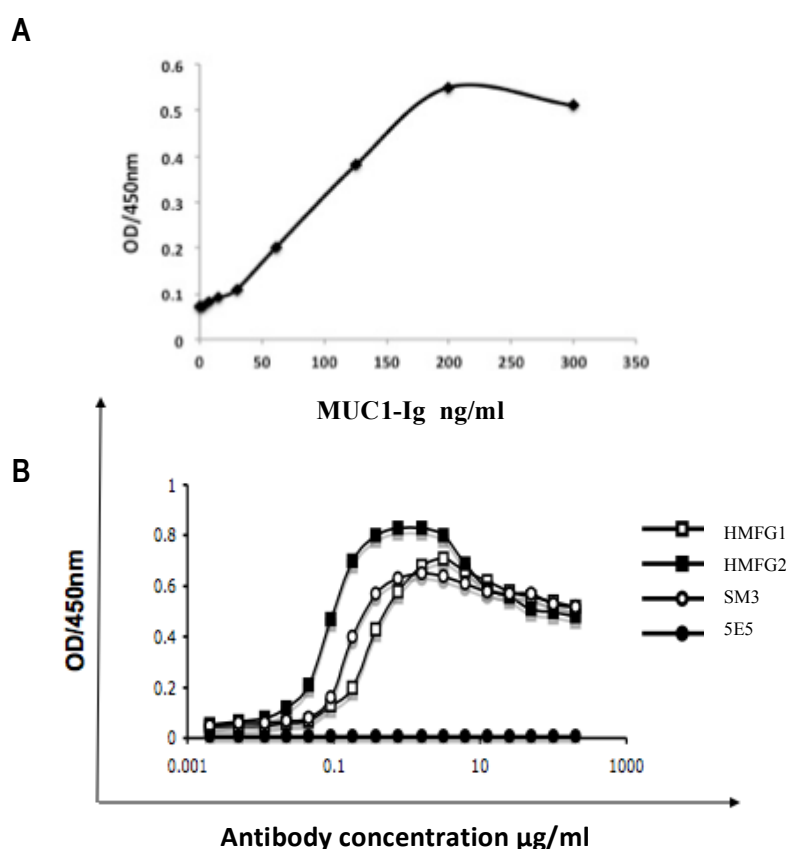


Figure 3.3 ELISA testing of MUC1 without O-linked glycans after EK cleavage

A: HMFG1 sandwich ELISA of purified MUC1-Ig without O-linked glycans (used as a standard), using 50 ng per well of purified HMFG1 as a capture antibody, and 1  $\mu\text{g}$  per well biotinylated HMFG1 as a detection antibody. B: ELISA titration, of various MUC1 MAbs using MUC1 without O-linked glycans as target coated plates (1  $\mu\text{g}$ /well).



Table 3.2: Amino acid analysis of recombinant MUC1-16TR without O-linked glycans

<i>Amino acid</i>	<i>Theoretical value (% by frequency)</i>	<i>ldlD MUC1 Naked (mol %)</i>
<b>Asx</b>	6.2	7.7
<b>Thr</b>	14.6	12.8
<b>Ser</b>	12.6	12.9
<b>Glx</b>	1.7	4.5
<b>Pro</b>	21.2	17.7
<b>Gly</b>	9.8	22
<b>Ala</b>	17.3	16.2
<b>Val</b>	5.5	5.6
<b>Met</b>	0.2	0
<b>Lle</b>	0	0.5
<b>Leu</b>	1	1.7
<b>Tyr</b>	0	0
<b>Phe</b>	0	0
<b>His</b>	4.5	6.1
<b>Lys</b>	0.8	1.2
<b>Arg</b>	4.0	3.5
<b>Trp</b>	0.2	0
<b>Cys</b>	0	0

### 3.3.2 Purification of MUC1-Tn

As with the production of unglycosylated MUC1 engineered CHO ldlD cells transfected with the recombinant MUC1 were used. However, by culturing the cells in medium containing GalNAc, MUC1 carrying only Tn was produced. ldlD-16TR-MUC1-Ig mutant cells were cultured in the presence of GalNAc in bioreactors and the supernatant concentrated and re-buffered as previously described, before being sent to our lab. The IgG portion was cleaved by 48 hours of enterokinase treatment (see materials and methods), and purification carried out by affinity chromatography, using the 5E5 mAb (specific for Tn and STn carried only on MUC1) bound to sepharose beads. Bound material was eluted from the column using 0.2 M glycine pH 2.5, and the eluted fractions tested in a 5E5 sandwich ELISA to determine which

fractions contained the eluted material. SDS PAGE followed by silver staining and Western blotting (fig 3.4) was then carried out to analyze the purified material.

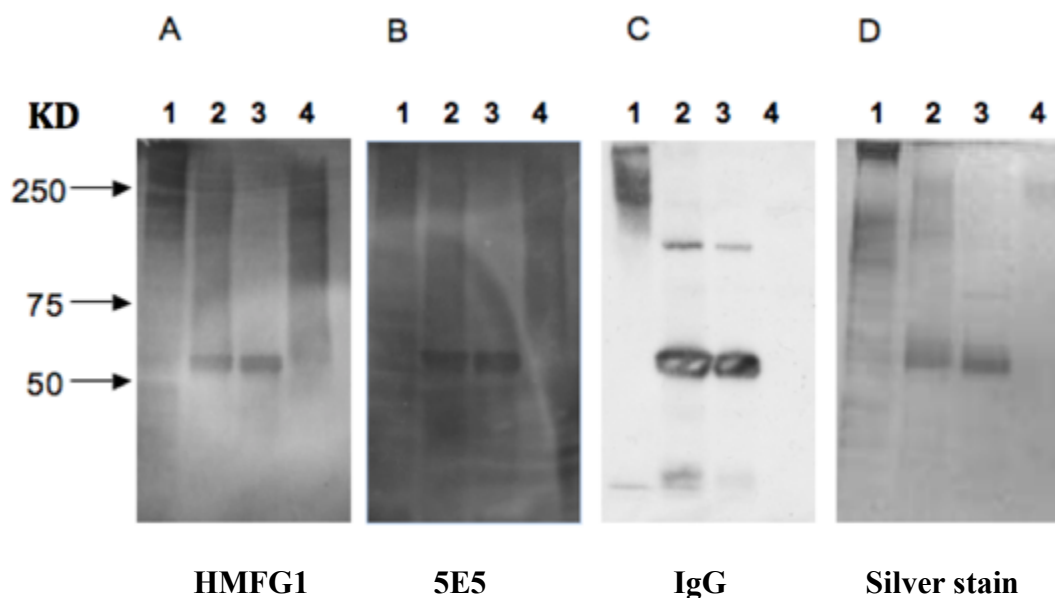
In figure 3.4A (HMFG1 Western blot) the majority of MUC1-IgG appears to migrate on a SDS PAGE gel at around 250 kDa, however a large smear is observed (lane 1). After EK cleavage (lane 2) a diffuse band is observed ranging from around 200 to 55 kDa. This appears to be the MUC1-Tn migrating as a diffuse band possibly due to variation in the number of sites glycosylated within each of the TR. A single band is notable at about 55 kDa, which is the migratory size for the MUC1-16TR without O-linked glycans. The 55 kDa single band could be MUC1-16TR without O-linked glycans, while the diffused band is believed to be the MUC1-Tn. In lane 3 (flow-through), the single band of 55 kDa range is present, indicating the presence of MUC1-16TR without O-linked glycans that was not retained by the 5E5 column. The eluted EK cleaved MUC1-Tn was identified by 5E5 sandwich ELISA, and a sample from the fraction containing the largest amount was run in lane 4.

Figure 3.4B (5E5 Western blot) is very similar to the HMFG1 Western blot, however as a band of about 55 kDa is also present in lane 3 it was concluded that a portion of the MUC1-Tn had very few glycosylation sites that did not affect its migration on a SDS PAGE gel. Moreover, this low level of glycosylated MUC1-Tn is not retained by the 5E5 column but is recognised by 5E5 on the Western blot.

Figure 3.4C (anti-IgG Western blot) also indicates that the majority of MUC1-Tn-IgG appears to migrate on a SDS PAGE gel at around 250 kDa (lane 1), that the majority of the material is cleaved after EK treatment, as a major band at around 55 kDa is present in lane 2, and that the Fc region of mouse IgG-2a is not retained by the 5E5 column, as a major band is also present in lane 3 (flow-through) and absent from the eluted material (lane 4).

Silver stain was used to assess the purity of the MUC1-Tn (fig 3.4D). A band at the very top of the gel was observed in the starting material, with additional weaker bands throughout (lane 1). After EK cleavage and in the flow-through, a band of around 55 kDa was seen with additional bands in the EK cleavage lane. However, only a very weak diffused band running around 250 kDa was observed in the MUC-

Tn fraction. Silver ions are reduced to insoluble silver metal granules in the vicinity of the protein molecules but not proteins carrying glycans, thus as expected MUC1-Tn only stained weakly. No other bands were observed, highlighting its good level of purity.



**Figure 3.4** Purification of MUC1-Tn from CHO-ldlD cells

*Purification stages of MUC1-Tn produced in CHO ldlD cells cultured in the presence of GalNAc using a 5E5 affinity column: Lane 1, MUC1-Tn-Ig; lane 2, MUC1-Tn after enterokinase cleavage treatment; lane 3, column flow-through; lane 4, pooled fraction 7-9 after elution with 0.2 M glycine pH 2.5. A: HMFG1 Western blot B: 5E5 Western blot C: Anti-IgG Western blot. D: Silver Staining.*

The concentration and purity of MUC1-Tn was determined by amino acid analysis (table 3.3), after extensively dialysing the glycoprotein against PBS to remove the glycine. The results indicate a very high level of purity. Mass spectrometry performed by our Swedish collaborator determined that an average 3.8 of the possible 5 sites in each TR were glycosylated on the recombinant MUC1-Tn. Thus, as GalNAc molecular weight is 203 Dalton, it was possible to estimate that about 11% of the total weight of recombinant MUC1-Tn-Ig was due to the sugars, or in other words, that about 60% of the total weight was made up by the glycosylated MUC1-Tn. Indeed about 0.6mg of MUC1-Tn from each mg of starting material was purified.

Table 3.3: Amino acid analysis of MUC1-Tn purified product

<i>Amino acid</i>	<i>Theoretical value (% by frequency)</i>	<i>Id1D MUC1 Tn (mol %)</i>
<b>Asx</b>	6.2	7.1
<b>Thr</b>	14.6	14
<b>Ser</b>	12.6	12.2
<b>Glx</b>	1.7	2.6
<b>Pro</b>	21.2	20
<b>Gly</b>	9.8	9.2
<b>Ala</b>	17.3	16
<b>Val</b>	5.5	6.5
<b>Met</b>	0.2	0.2
<b>Lle</b>	0	0.1
<b>Leu</b>	1	1.2
<b>Tyr</b>	0	0
<b>Phe</b>	0	0
<b>His</b>	4.5	6.2
<b>Lys</b>	0.8	0.9
<b>Arg</b>	4.0	3.3
<b>Trp</b>	0.2	0
<b>Cys</b>	0	0

### 3.3.3 Purification of MUC1-ST

The concentrated supernatant from wild-type CHO cells transfected with MUC1-Ig was treated with enterokinase to remove the Ig domain (see materials and methods). The effectiveness of this treatment was assessed by running the supernatant on an SDS PAGE gel followed by silver stain for protein, which stains Ig but not MUC1-ST (fig 3.5A), and Alcian blue for sialylated glycans, as the dye stains acid mucopolysaccharides and glycosaminoglycans, thus staining MUC1-ST but not IgG (fig 3.5B). Following the cleavage of the Ig portion, MUC1 was purified using anion exchange chromatography, which was preferentially chosen as a large scale purification method as the conditions for MUC1's purification have previously been developed (Backstrom M. *et al.* 2003). Negatively charged proteins were retained by the column and are eluted by increasing the ionic strength of the buffer, thus separating MUC1-ST glycoprotein from other contaminants (fig 3.5C). Collected fractions were run on SDS PAGE and sialic acid bearing proteins identified by

Alcian blue staining and the purity of each fraction was determined by silver staining (fig. 3.5D).

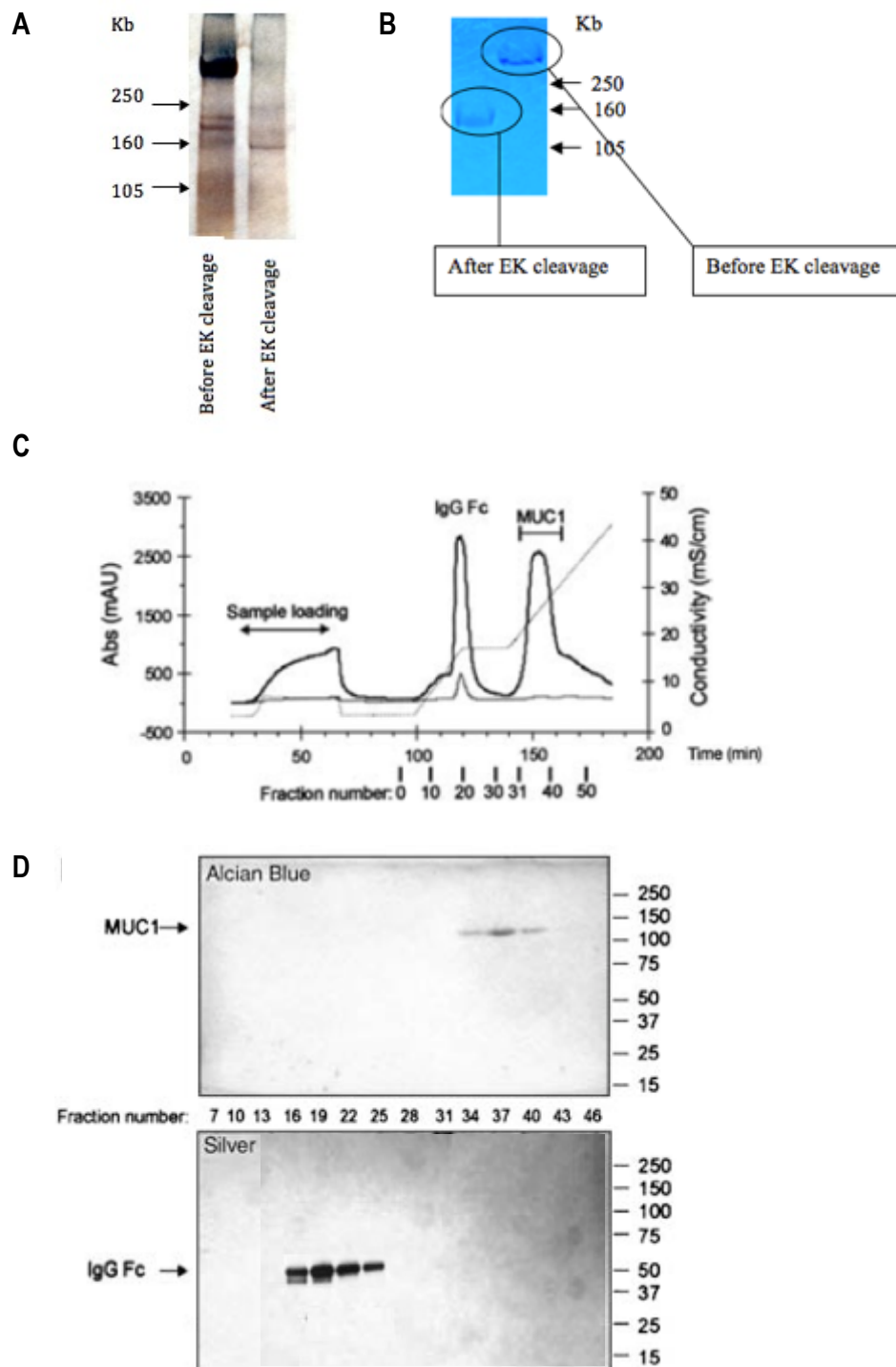


Figure 3.5. Purification of MUC1 ST from WT CHO cells

A, Silver stain; Removal of Ig region from ST-MUC1-IgG from CHO K1 cells via enterokinase cleavage results in loss of silver staining. B, Alcian blue; Removal of Ig region of ST-MUC1-IgG from CHO K1 cells via enterokinase cleavage results in a size 'shift' on Alcian blue staining. C; HPLC trace. D; Alcian blue and silver staining of ST-MUC1 from HPLC fractions collected after elution.

Fractions that stained for Alcian blue, and which were virtually clear of background silver staining, were pooled, dialysed against water and then lyophilised. The weight was calculated after lyophilisation, and 20 µg sent for amino acid analysis for a more precise quantity and quality measure (table 3.4 and materials and methods 2.14). The quantity of MUC1-ST purified was as expected by its molecular weight (ratio between predicted unglycosylated versus glycosylated) to be 0.7 mg purified from each mg of starting material.

*Table 3.4: Amino Acid analysis of purified MUC1-ST*

<i>Amino acid</i>	<i>Theoretical value (% by frequency)</i>	<i>CHO MUC1 ST (mol %)</i>
<b>Asx</b>	6.2	7.2
<b>Thr</b>	14.6	13.3
<b>Ser</b>	12.6	12.4
<b>Glx</b>	1.7	3.5
<b>Pro</b>	21.2	18
<b>Gly</b>	9.8	9.5
<b>Ala</b>	17.3	16
<b>Val</b>	5.5	6.4
<b>Met</b>	0.2	0.2
<b>Lle</b>	0	0.6
<b>Leu</b>	1	2.1
<b>Tyr</b>	0	0.5
<b>Phe</b>	0	0.5
<b>His</b>	4.5	3.9
<b>Lys</b>	0.8	1.4
<b>Arg</b>	4.0	4
<b>Trp</b>	0.2	Not available
<b>Cys</b>	0	Not available

### 3.3.4 Purification of MUC1-T

To produce MUC1-T, the MUC1-ST was treated with neuraminidase-coated agarose beads to remove the sialic acid as described in material and methods. The resultant MUC1 was checked by Alcian blue and Western blot. For the Western blot, two lectins specific for particular glycans were used for detection: peanut agglutinin (PNA) specific for Gal- $\beta$ 1-3-GalNAc the T glycan and Maackia amurensis hemagglutinin (MAA) specific for  $\alpha$ 2-3 linked sialic acid. Figure 3.6 shows that after neuraminidase treatment PNA staining is drastically increased with a smear type of staining reflecting the variable migration on a SDS PAGE gel, probably due to the variable glycosylation of MUC1-T. MAA staining was found to be weak, however, the disappearance of the band on the neuraminidase treated glycoprotein further indicate the removal of the  $\alpha$ 2-3 linked sialic acid.

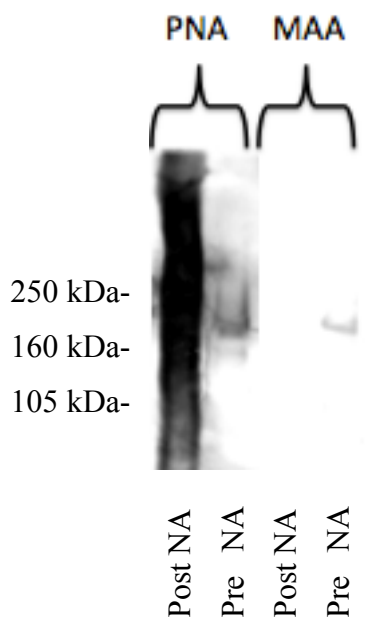


Figure 3.6. CHO K1 MUC1 PNA and MAA Western blot

PNA and MAA lectin Western blots of MUC1-ST from CHO K1 cells after purification and neuraminidase treatment (NA).

### 3.3.5 Purification of MUC1-STn

Material derived from large scale production of MUC1 secreted by CHO-K1 cells transfected not only with the MUC1 construct, but also with a construct containing the ST6GalNAc-I cDNA, resulted in secreted MUC1 expressing mainly the glycan STn (Sewell R. *et al.* 2006). To purify MUC1-STn the Ig portion was cleaved off using 48 hours of enterokinase treatment (Fig 3.7A). The purification of the

concentrated secreted material was achieved by affinity chromatography, using 5E5 mAb (specific for Tn and STn only on MUC1) bound to sepharose beads. Bound material was eluted from the column using 0.2 M glycine pH 2.5, and the quantity calculated by 5E5 mAb sandwich ELISA using MUC1-STn-Ig as a standard (fig 3.7B). Unfortunately, the yield of purified materials was extremely low, with only 50 µg of protein being purified from each mg of starting materials, probably due to weaker affinity to MUC1-STn as compare to MUC1-Tn of the 5E5 Abs (fig 3.7C). In the future it may be possible to improve the yield by using either HMFG1 or HMFG2 antibodies bound to sepharose beads or by ion exchange chromatography.

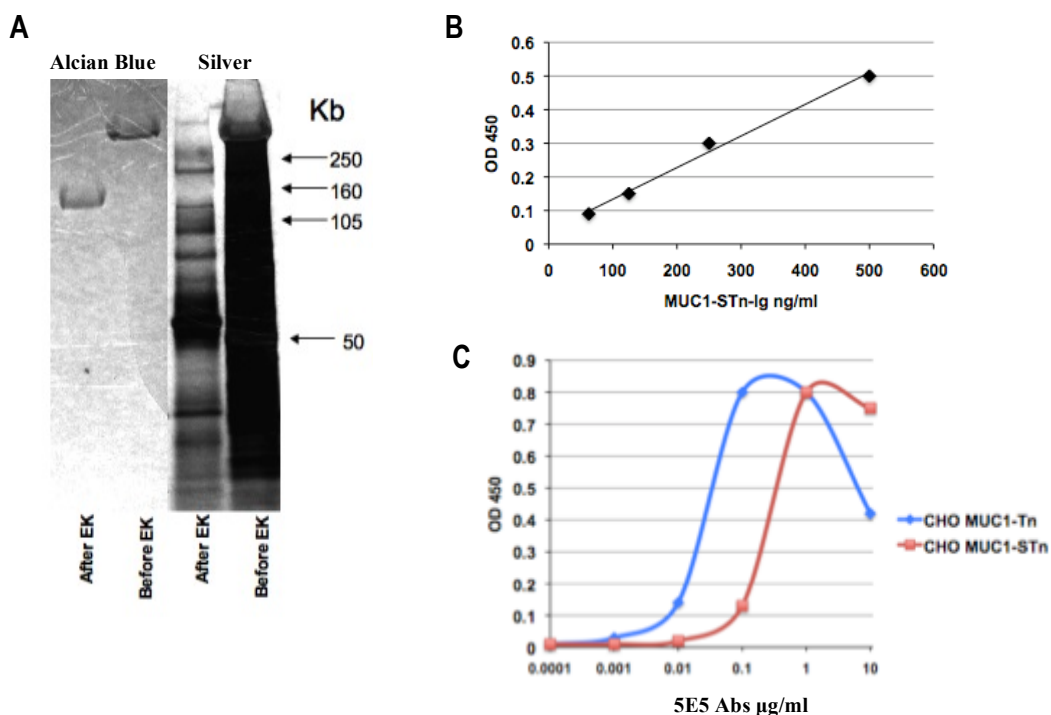


Figure 3.7. Purification of MUC1 STn

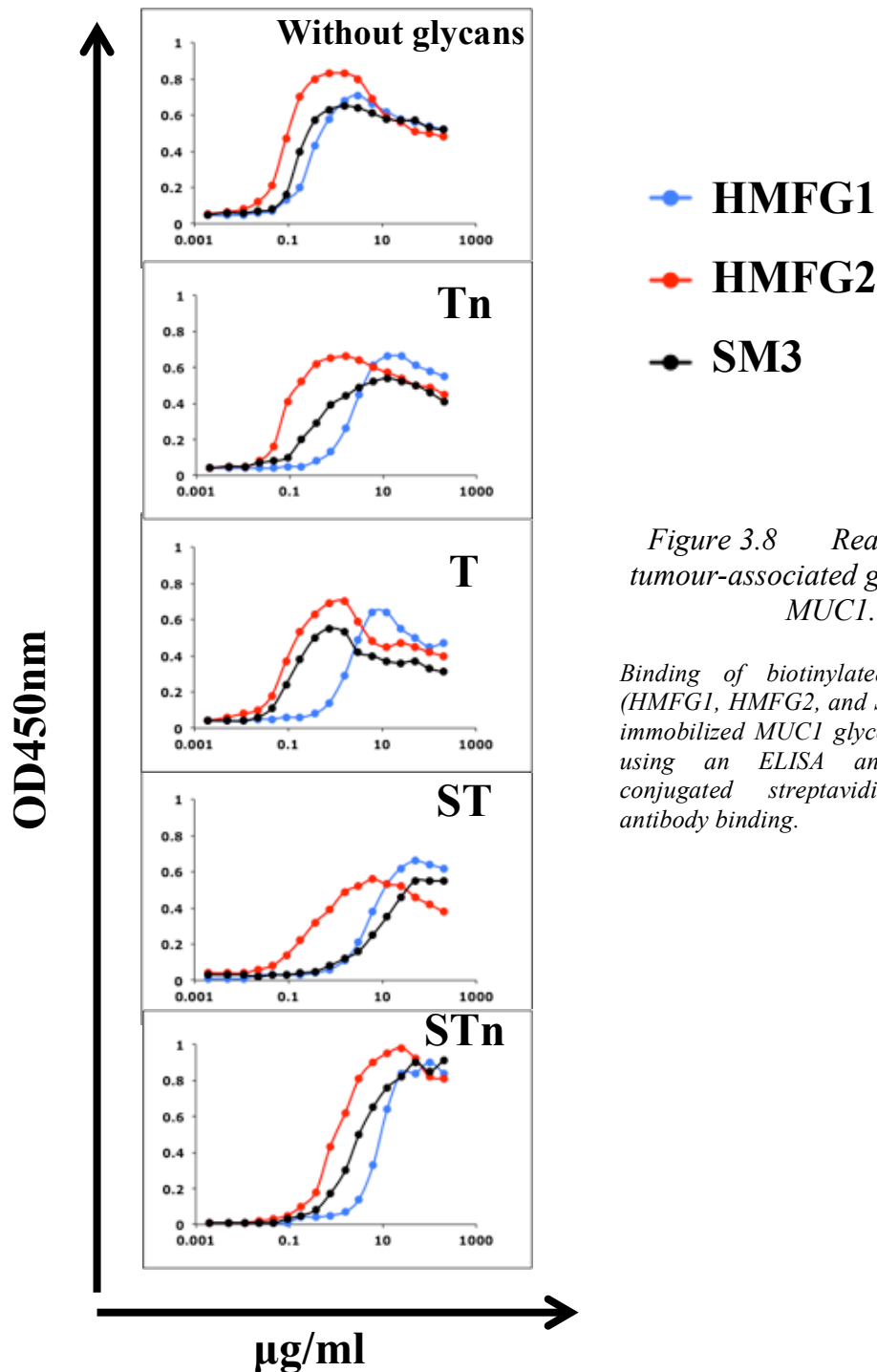
A; Removal of Fc region from STn-MUC1-Ig expressed by CHO K1-ST6GalNAc-I cells via enterokinase cleavage results in loss of silver staining of the major band and in a size 'shift' seen on Alcian blue staining. B; MUC1-STn-Ig standard using 5E5 sandwich ELISA. C; 5E5 ELISA titration on plate coated with 1 µg per well of either MUC1-Tn-Ig or MUC1-STn-Ig.

### 3.3.6 Binding of MUC1 MAbs to the CHO produced MUC1 glycoproteins

In order to further characterize the purified MUC1 glycoforms, the reactivity of three MUC1 antibodies to each of the glycoforms was determined (fig 3.8). Of the panel of antibodies used HMFG1, HMFG2 and SM3 are known to react with peptide epitopes within the tandem repeat of MUC1, although their binding is influence by the glycans. HMFG1 reacts with MUC1 with long glycans but its binding is inhibited by



sialic acid (Burchell J. and Taylor-Papadimitriou J. 1993). HMFG2 binding to MUC1 is reduced by long glycan chains (Burchell J. and Taylor-Papadimitriou J. 1993) while SM3 binding is blocked by core 2 glycans (Dalziel M. *et al.* 2001, Mungul A. *et al.* 2004). All three antibodies were shown to bind to all four forms of MUC1. However the antibody 5E5 binds only to MUC1-Tn and MUC1-STn (fig 3.3 and 3.7C).



*Figure 3.8 Reactivity with tumour-associated glycoforms of MUC1.*

*Binding of biotinylated MUC1 Abs (HMFG1, HMFG2, and SM3) to specific immobilized MUC1 glycoforms detected using an ELISA and peroxidase-conjugated streptavidin to detect antibody binding.*

### 3.3.7 Endotoxin testing

Sterile techniques were applied throughout the purification procedures; however, there is always the possibility of pathogenic contamination, therefore before any immunological studies were carried out, all MUC1 recombinant glycoproteins were tested for endotoxins. Following purifications, endotoxin testing was performed using the LAL-1000 kit from Lonza as described in materials and methods, and only batches with < 5 EU/ml of endotoxin per  $\mu\text{g}$  of protein were used for further studies (fig. 3.8 A and B).

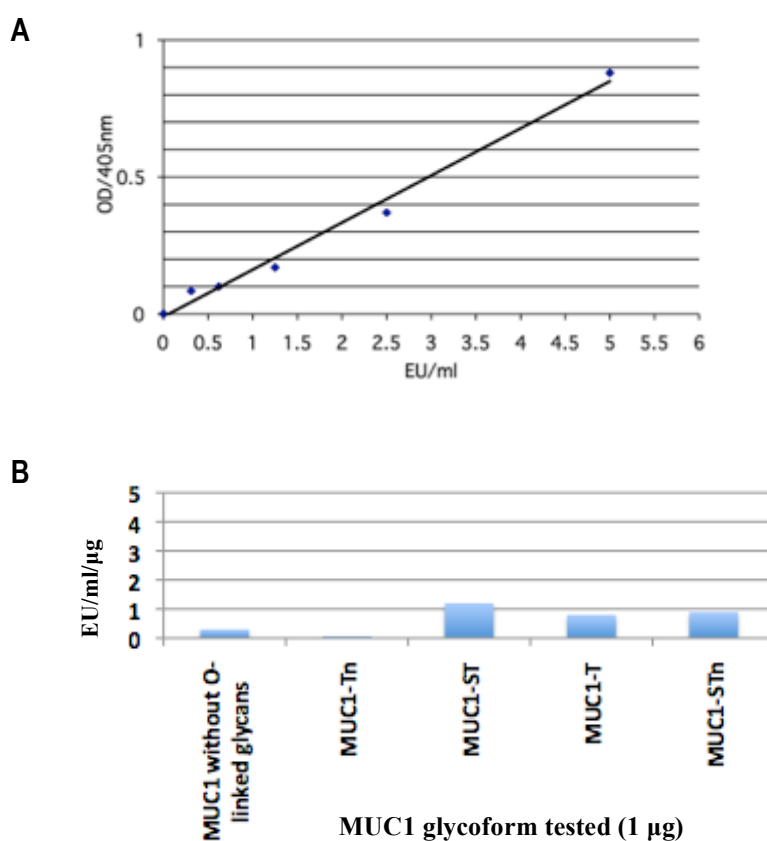


Figure 3.9 Endotoxin testing

A; Standard curve of endotoxin test using the LAL-1000 assay. B; examples of purified MUC1 glycoforms tested at a concentration of 1  $\mu\text{g}/\text{ml}$

### 3.4 Discussion

In this chapter the purification of MUC1 glycoforms carrying the specific tumour-associated glycans Tn, Sialyl-Tn, T and Sialyl-T has been described. The glycoforms were characterised by the following methods:

1. Their binding to four monoclonal antibodies to MUC1 was determined. These antibodies showed differential binding to the different glycoforms with 5E5, which is specific to MUC1 carrying Tn and STn, only binding the MUC1-Tn and STn glycoforms.
2. The purity of the glycoforms was determined by silver staining of SDS polyacrylamide gels and amino acid analysis.
3. All the batches of the different glycoforms were analysed for endotoxin contamination.
4. The glycosylation status was initially analysed by Alcian blue and lectin staining of material run on polyacrylamide gels and the precise nature of the glycans determined by mass spectrometry by the Hansson Lab in Sweden (Backstrom M. *et al.* 2003, Link T. *et al.* 2004).

MUC1 has been widely studied in the cancer context; indeed it is estimated that 75% of all solid tumours express MUC1 (Tang C. K. and Apostolopoulos V. 2008), and that MUC1 positive cancers account for the majority of cancer deaths (Greenlee R. T. *et al.* 2001). MUC1 is aberrantly glycosylated and overexpressed in 90-96% of breast carcinomas (Burchell J. *et al.* 1987, Girling A. *et al.* 1989, Rakha E. A. *et al.* 2005). The cancer-associated change in the O-linked glycans carried by MUC1 exposes predominantly the glycans Tn, Sialyl-Tn, T, Sialyl-T and these are the glycoforms produced in this chapter. However, other O-linked glycans can be found in breast cancers although it is unclear if these can be carried on MUC1 (Julien S. *et al.* 2011).

This aberrant glycosylation results in the MUC1 mucin expressed by carcinomas being antigenically distinct from that expressed by normal mammary epithelial cells as it been shown to induce immune responses. Autoantibodies (IgG and IgM) to cancer-associated MUC1 are common in breast cancer patients and increased levels of these antibodies have been correlated with increased survival in breast cancer (von Mensdorff-Pouilly S. *et al.* 1996; von Mensdorff-Pouilly S. *et al.* 2000b).

Furthermore, antibodies to MUC1 tumour-associated glycoforms found in the sera of early breast cancer patients are significantly associated with reduced incidence of and increased time to metastasis (Blixt O. *et al.* 2011). However, at present the contribution of each specific glycan to cancer and their immunogenicity is poorly understood.

In collaboration with a European Consortium led by our group we have generated a mammalian expression system for the production of recombinant MUC1 in CHO-K1 or CHO *ldld* mutants. Chinese hamster ovary (CHO) cells were chosen because these cells are the most widely used mammalian cells for recombinant protein expression and large-scale production. They provide stable and accurate glycosylation, offering a post-translationally modified product and thus a more accurate *in vitro* rendition of the natural protein (Sheeley D. M. *et al.* 1997, Werner R. G. *et al.* 1998). In our system, it was found that CHO cells and their derivatives are powerful tools for the preparation of glycan-specific MUC1 glycoproteins.

With regard to unglycosylated and the Tn (GalNAc) carrying MUC1 glycoprotein, their production was made possible by exploiting the deficiency of UDP-Gal/UDP-GalNAc 4-epimerase present in the *ldld* mutant CHO cells (Kingsley D. M. *et al.* 1986), which leaves these cells unable to synthesise galactose and GalNAc. The process can be reversed by providing the cells with exogenous sources of sugars, which are utilized via a salvage pathway that does not require participation of the 4-epimerase enzyme lacking in these cells (Segal S. 1983). Thus, the *ldld* mutant when transfected with MUC1 (16TR)-IgG construct, secreted unglycosylated MUC1. This was confirmed by the migratory size of the product on an SDS/PAGE gel and the absence of staining by the monoclonal antibody 5E5, which specifically recognizes MUC1-Tn. The *ldld* mutant transfected with the MUC1 (16TR)-IgG construct grown in the presence of GalNAc secreted MUC1 carrying only the Tn antigen. This had an average site occupancy of 3.4 out of a possible 5 for each tandem repeat, and as expected by the type of purification (affinity chromatography using 5E5 mAb), the material bound to the 5E5 mAb.

MUC1 produced in wild-type CHO K1 cells and purified by ion exchange chromatography was found to carry mostly the glycan sialyl T (84.3%), or disialyl T

(13.3%), with only 1.9% T as determined by the Hansson lab (Backstrom M. *et al.* 2003). Because of this predominance of the sialyl T glycan, we have considered this glycoprotein as being virtually pure MUC1 sialyl T, with a similar O-glycosylation pattern to that of MUC1 from some breast cancer cell lines, like the extreme case of the human metastatic breast cancer cell line T47D which only express MUC1-T and sialyl T (Backstrom M. *et al.* 2003), and many primary breast cancers (see appendix I). Furthermore, the site occupancy of the MUC1 sialyl T glycoprotein was found by the Hansson lab to be on average 4.3 glycosylated sites out of a possible 5 per tandem repeat, which is similar to that previously reported for endogenous MUC1 from the breast carcinoma cell line T47D (4.8 per TR) (Muller S. *et al.* 1999). Speculatively, the small difference could possibly be due to the presence of the Fc tail in the construct, which may induce faster transit time through the Golgi, preventing the complete action of the polypeptide-GalNAc-transferases that initiates the synthesis of the O-glycosylation. On the other hand, different cell lines differ in their glycosylation ability and have variable levels of polypeptide GalNAc-transferases.

A very effective way to remove the sialylation on proteins is by treating them with neuraminidase (Backstrom M. *et al.* 2003), and so this method was used on the purified MUC1-ST produced in CHO K1 cells. The neuraminidase treated MUC1 glycoprotein carried only the glycan T (Gal  $\beta$  1-3 GalNAc), which was confirmed by the absence of MAA staining (Western Blotting), which selectively stains  $\alpha$ 2-3 sialic acids and the increase in PNA staining.

CHO K1 cells co-transfected with MUC1-16TR-IgG, and ST6GalNAc-I secreted mostly sialylated GalNAc (83%) with a small amount of disialyl T (12%), and sialyl T (4%) (Professor Gunnar Hansson personal communication). The purification of this glycoform has shown that MUC1-ST<sub>n</sub> can be purified by affinity chromatography using the 5E5 mAb which has been reported to cross-react with MUC1-ST<sub>n</sub> (Tarp M. A. *et al.* 2007). However, the yield was deemed to be very poor, with only 5% of product being retained on the column, leaving considerable room for improvement. Perhaps by using other MAbs such as HMFG1 or HMFG2 in

an affinity chromatography system or by using an anion exchange chromatography system it may be possible to improve MUC1-ST<sub>n</sub> yield.

The availability of these pure glycoforms of MUC1, carrying defined and specific sugars, gives us an opportunity to investigate whether any of these MUC1 glycoforms can provide protective immunity against cancer, and to investigate how specific glycans of MUC1 interact with lectin receptors of the immune system. This will be described in the following chapter.

# ***Chapter 4***

***In vivo immune response to MUC1 glycoforms***

## 4.1 Introduction

To address the hypothesis that specific glycoforms of MUC1 can induce immune responses, the MUC1 glycoforms produced in chapter 3 were used to measure their effectiveness in eliciting immune responses in MUC1 transgenic mice. It was hypothesized that the repetitive nature of the glycans carried on MUC1 tandem repeats could be similar to that observed in pathogen-associated molecular patterns and therefore may induce an immune response.

In a project conducted by the USA's National Cancer Institute MUC1 was listed second most optimal immunotherapeutic target out of 75 candidates (Cheever M. A. *et al.* 2009). The characteristics that make MUC1 such a good target for immunotherapy include over-expression in carcinomas the repetitive nature of the glycans and aberrant O-linked glycosylation as compared to normal epithelial cells. Moreover, there is increasing evidence that MUC1 is involved in tumour growth (Mungul A. *et al.* 2004, Raina D. *et al.* 2009). Preclinical studies using MUC1 transgenic mouse models offer the best preclinical models to evaluate the effectiveness of immune responses at present. However, even though the MUC1 transgenic mouse model allows evaluation of MUC1 immunogens as self-antigens, the response relies on the mouse immune system, which undoubtedly has differences from the human system.

Preclinical studies using MUC1-Tn or MUC1-STn peptides have already uncovered the immune system's ability in eliciting strong humoral responses against cancer-associated glycoforms of MUC1 in MUC1 transgenic mice (Sorensen A. L. *et al.* 2006). Various glycans abundantly expressed in the cancer-associated form of MUC1, have been shown to bind receptors present on cells of the immune system (Van Gisberger K. P. *et al.* 2005, Napoletano C. *et al.* 2007). Furthermore, antigen presenting cells can process MUC1 expressing simple O-linked glycans and presentation on both MHC classes I and II is possible (see chapter 1.11, Lakshminarayanan V. *et al.* 2012).



## 4.2 Results

### 4.2.1 *In vivo immune response to MUC1 glycoforms*

In order to investigate if any of the various tumour-associated MUC1 glycoproteins were able to raise either cellular and/or humoral immunological responses to MUC1, transgenic mice for human MUC1 (Peat N. *et al.* 1992) were immunized with various tumour-associated MUC1 glycoproteins, purified as described in chapter 3. The immunization consisted of three subcutaneous injections, given at three week intervals, of either 10 µg of unglycosylated, Tn, ST or T MUC1 glycoproteins emulsified in Freund's complete adjuvant for the first immunization, then Freund's incomplete for the following two (see Freund's adjuvant in materials and methods). Seven days after the last immunization, a single cell suspension was prepared from the spleens and splenocytes stimulated *in vitro* with the same immunogen for 52 hours before assaying the supernatant for cytokine production (see materials and methods). As notable in figure 4.1, the only glycoprotein that was able to stimulate a cellular immunological response as determined by IFN-γ secretion was MUC1-T.

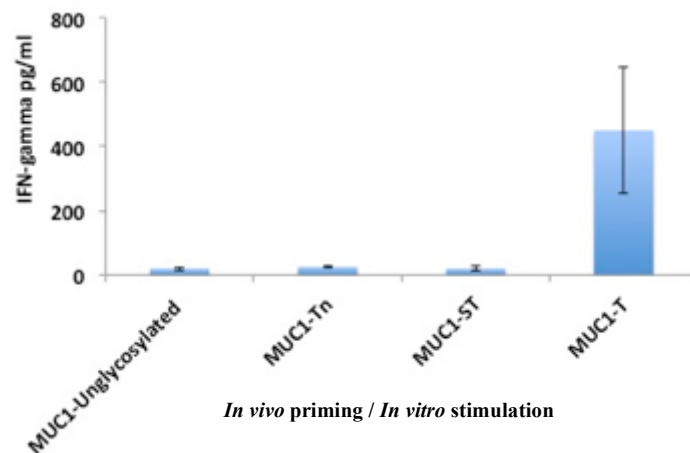


Fig 4.1: Cellular response to various MUC1 glycoproteins

Single cell suspension of splenocytes from MUC1 transgenic mice immunized with various purified MUC1 glycoproteins (10 µg per injection x 3) were incubated with the same MUC1 glycoprotein *in vitro* (10 µg/ml) for 52 hours. The supernatant was then analyzed for IFN-γ production by ELISA. The results shown are the average of 4 mice per group.

#### 4.2.2 Immune response to MUC1-T

The reproducibility of the cellular response present in MUC1 transgenic mice immunized with MUC1-T was assessed by repeating the experiment four times (n=12). Moreover, different purifications of MUC1-T were used in order to eliminate the possibility of a batch specific contaminant. The number and frequency of MUC1-T immunization were also optimized (fig 4.2). The results found that MUC1-T was capable of consistently eliciting immune responses, measured by IFN- $\gamma$ , that the response was not batch specific, and that three 10  $\mu$ g immunization given at three weekly intervals was the best tested priming protocol for MUC1-T response.

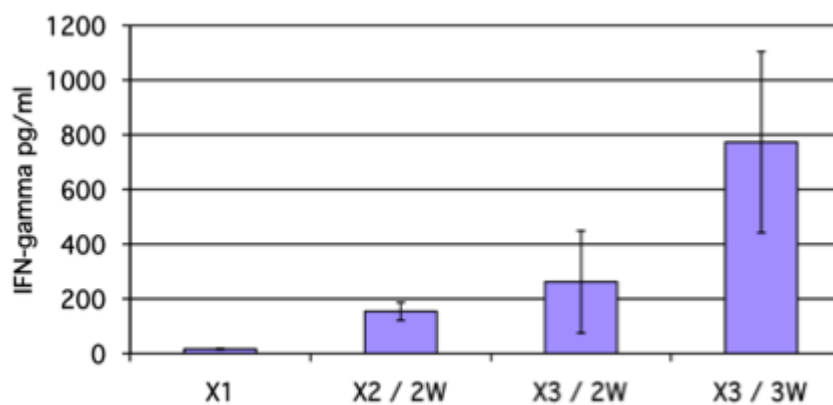
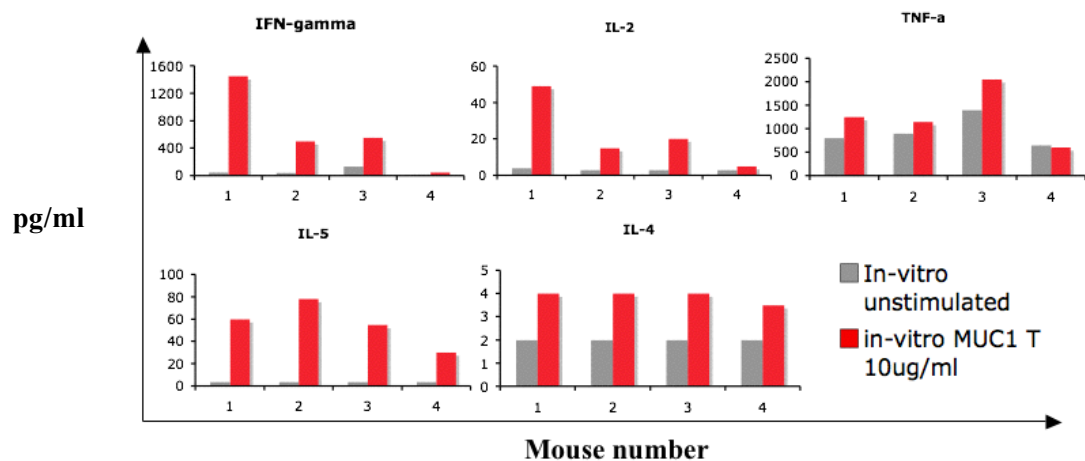


Fig 4.2: Optimizing the number and intervals for CHO MUC1-T immunization

Four MUC1 transgenic mice per group were immunized with 10  $\mu$ g of MUC1-T either once (X1), twice at two weekly intervals (X2 / 2W), three times at two weekly intervals (X3 / 2W), or three times at three weekly intervals (X3 / 3W). Seven days after the last immunization single cell suspensions of splenocytes from the immunized mice were incubated *in vitro* with 10  $\mu$ g/ml of MUC1-T for 52 hours prior to ELISA analysis for IFN- $\gamma$  production. The results shown are the average IFN- $\gamma$  level from each group (n=4) and their standard deviation.

In general a Th1-type of response tends to produce a pro-inflammatory response responsible for killing and for perpetuating immune responses, while Th2 responses are associated more with anti-inflammatory responses and so can counteract excessive pro-inflammatory responses that can lead to uncontrolled tissue damage. The optimal scenario would therefore be the one that produces a well balanced Th1 and Th2 response, to allow maximal T cell efficacy (Sallusto F. *et al.* 2004). With that in mind, we investigated the secretion of several cytokines that are known to be unique to the Th1 type of response (IFN- $\gamma$ , TNF- $\alpha$  and IL-2) or to the Th2 type of response (IL-4 and IL-5). Figure 4.3 represents examples of responses in four mice

immunized with three injections of 10 µg of MUC1-T, three weeks apart. After the final injection the splenocytes were removed and stimulated *in vitro* for 48 hours with 10 µg/ml of MUC1-T (see materials and methods). Three mice out of four elicited a relatively strong Th1 type of response indicated by the presence of IFN-γ and IL-2 in the media. Furthermore, the four investigated mice also appear to elicit a Th2 type of response as seen by the presence of IL-5 and IL-4 in the media. However, the Th2 response was weak as the amount of IL-4 secreted into the media was very low, so it can be concluded that for three mice out of four the response generated is predominantly Th1. In addition, as seen with mouse number four, the generated Th2 type of response is weak even in mice that do not elicit a strong Th1 type of response.



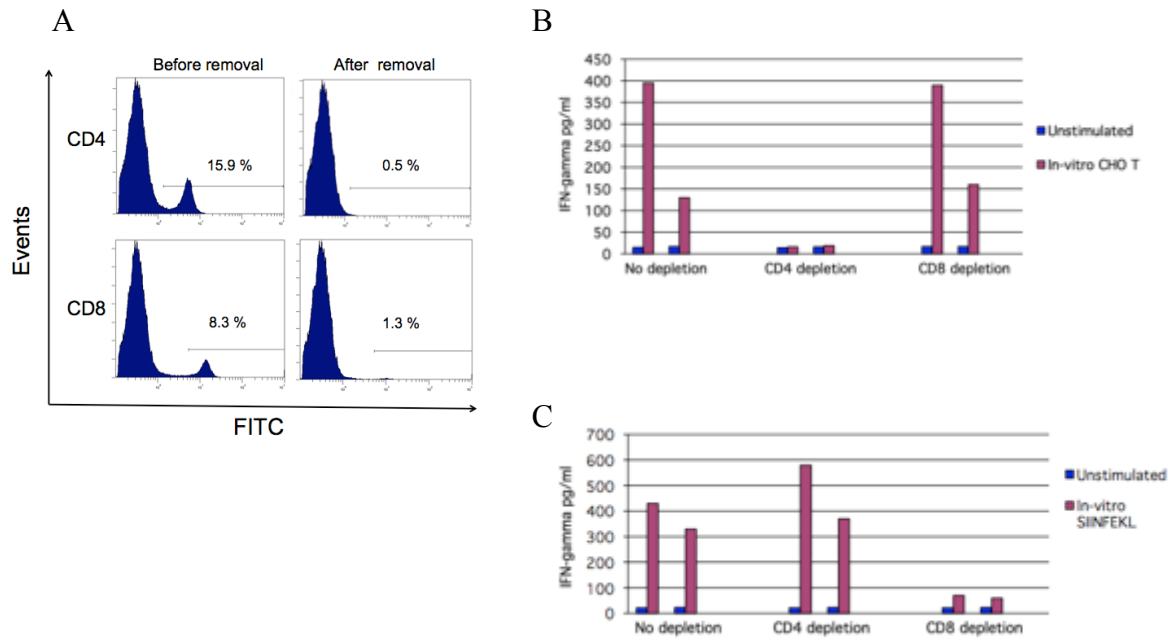
**Fig 4.3:** *MUC1-T induces mainly a Th1 type of response*

TH1/TH2 CBA (Cytometric Bead Array) analysis of various cytokines secreted into the culture media from splenocytes stimulated *in vitro* by 10 µg/ml of MUC1 glycoprotein carrying the T antigen (Galβ1-3GalNAc), following the *in vivo* priming (10 µg x 3) with MUC1-T. C57Bl/6 MUC1 TG mice (mice 1, 2, 3, and 4 represented).

### ***Identification of T cell subsets***

To establish which subset within the splenocytes was responding to MUC1-T, CD4 and/or CD8 T cells were depleted prior to *in vitro* stimulation. After immunization of the mice with MUC1-T, the CD8<sup>+</sup> or CD4<sup>+</sup> expressing cells were removed from splenocyte single cell suspension using CD4 or CD8 specific antibodies with the Amaxa system (see methods). The efficiency of depletion was measured by flow cytometry and as can be seen in figure 4.4A the vast majority of CD8<sup>+</sup> or CD4<sup>+</sup> T cells were removed. The depleted splenocytes were stimulated *in vitro* with 10 µg/ml

of MUC1-T glycoprotein for 52 hours prior to IFN- $\gamma$  secretion measurements (fig 4.4B). As a control for depletion of the T cells mice immunized with ovalbumen, and stimulated *in vitro* with the peptide SIINFEKL were included. This response is known to be of a Th1 type elicited through CD8 cells (Arnaboldi P. M. *et al.* 2009), as shown in figure 4.4C. The results presented in figure 4.4 clearly indicated that the response to MUC1 T requires CD4 expressing cells.

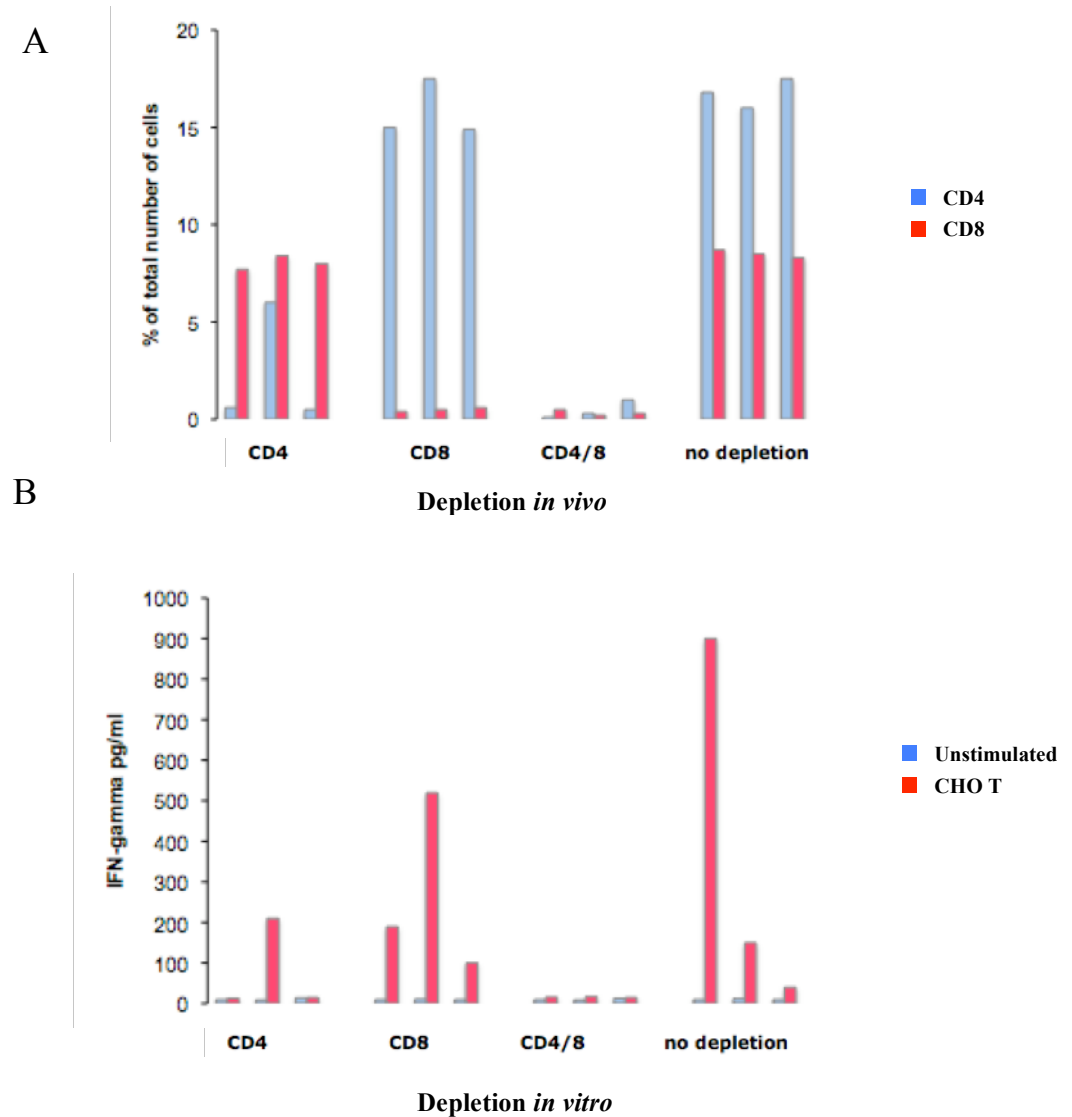


**Fig 4.4:** IFN- $\gamma$  responses following *in vitro* depletion of CD4/8 +ve T cells.

Splenocytes from MUC1 TG mice following *in vivo* priming (10  $\mu$ g x 3) with MUC1-T, or control (10  $\mu$ g x 3, ovalbumen), were depleted of CD4+ or CD8+ cells prior to *in vitro* stimulation with 10  $\mu$ g/ml of MUC1-T (Gal $\beta$ 1-3GalNAc) or SIINFEKL peptide. A: FACS analysis of spleen cells before and after CD4+ or CD8+ T cells removal, stained with FITC directly labelled anti CD4 or CD8 Abs. B: IFN- $\gamma$  secreted by stimulated splenocytes from MUC1-T primed mice, with or without T cell removal and with or without *in vitro* stimulation (N=2). C: ELISA for IFN- $\gamma$  in ovalbumen primed mice, with or without T cell removal and with or without *in vitro* stimulation with SIINFEKL (N=2).

In order to confirm that CD4 positive T cells were the sole cells responsible for the *in vivo* response, T lymphocytes expressing CD4 and/or CD8 were depleted *in vivo*, using monoclonal antibodies to CD4 and/or CD8 (see materials and methods) before and during vaccination with MUC1-T. Spleen cells from these mice were stimulated *in vitro* with MUC1-T for 52 hours before assaying the supernatant for cytokine production. Figure 4.5B, also shows that CD4 + cells are essential for the response to MUC1-T. One mouse out of three of the CD4 + *in vivo* depletion, appear to respond after *in vitro* stimulation. However, as illustrated in figure 4.5A *in vivo* depletion was variable, and in particular for the mouse that responded *in vitro* following priming

with MUC1-T, a considerable number of CD4<sup>+</sup> T cells were not depleted *in vivo*. With regard to the involvement of T lymphocytes expressing CD8, their *in vivo* depletion did not affect the response, leading to the conclusion that mainly CD4<sup>+</sup> cells are responding to MUC1-T glycoprotein immunization, and concord with the data derived by the CD4<sup>+</sup>/CD8<sup>+</sup> *in vitro* depletion analysis.

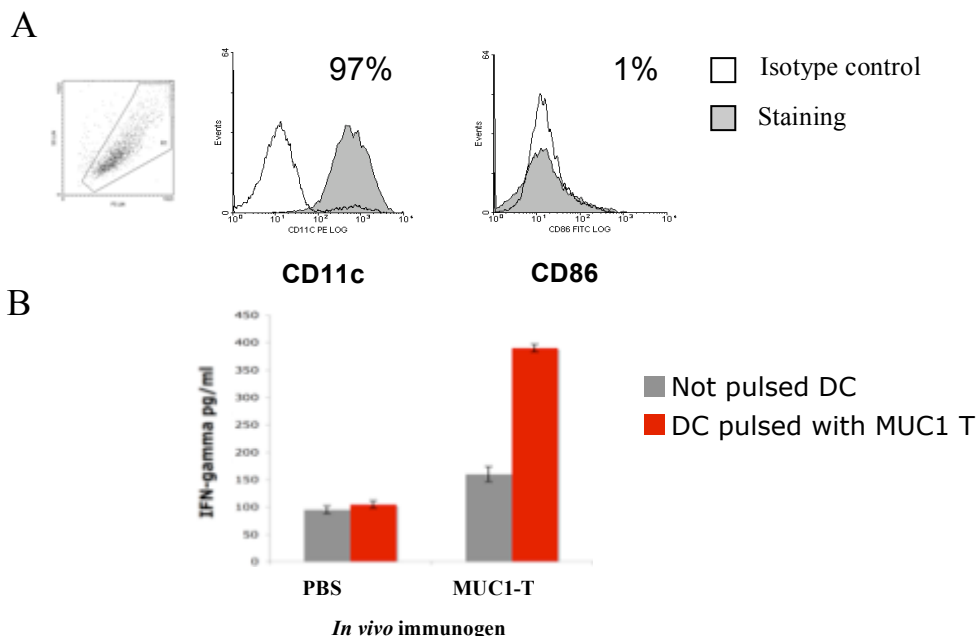


**Fig 4.5:** *IFN-γ* responses following *in vivo* depletion of CD4/8<sup>+</sup> T cells.

Splenocytes from MUC1 TG mice, *in vivo* depleted of CD4<sup>+</sup> and/or CD8<sup>+</sup> cells and *in vivo* primed (10 µg x 3) with MUC1-T were *in vitro* stimulation with 10 µg/ml of glycoprotein carrying the T antigen (Galβ1-3GalNAc). A: % of CD4 and CD8<sup>+</sup> cells following CD4 and/or CD8<sup>+</sup> depletion and MUC1-T immunization. B: ELISA for IFN-γ from splenocytes stimulated *in vitro* by 10 µg/ml of MUC1 carrying the T antigen (Galβ1-3GalNAc).

### 4.2.3 Processing and presentation of MUC1-T by DC

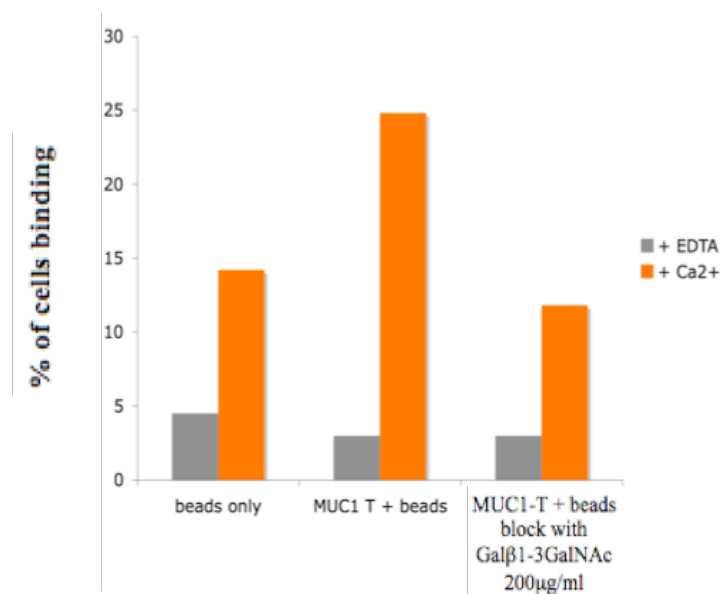
In order to understand the mechanisms involved in processing and presentation to T lymphocytes of MUC1-T epitopes, we used bone marrow derived dendritic cells (bmDC) to present MUC1-T epitopes *in vitro*. Adherent cells derived from mouse bone marrow were cultured for 7 days in the presence of GM-CSF, stained for the surface marker CD11c (DC marker) and CD86 (mature DC marker) and then analysed by flow cytometry. As illustrated in figure 4.6A most of the cultured cells expressed the surface marker CD11c, while not expressing the surface marker CD86, thus indicating a homogeneous immature DC population. Bone marrow derived immature DC were pulsed *in vitro* for six hours with recombinant purified MUC1-T (10 µg per million cells), washed in PBS, than added at a ratio of 10:1 (Spleen cells : DC) to single cells suspension from spleen of mice previously immunized with MUC1-T or from control mice. As shown in figure 4.6B, IFN $\gamma$  secretion was found in the supernatant of cells stimulated *in vitro* with bmDC pulsed with the MUC1-T. MUC1-T must therefore be taken up by the DCs, processed through the MHC class II antigen-presenting pathway and presented on the cell surface where it can stimulate the primed T cells.



**Fig 4.6:** *Presentation in vitro of MUC1-T by bmDC*

Splenocytes stimulated *in vitro* by bone marrow derived DC A: expression of the surface molecule markers CD11c (DC marker), and CD86 (mature DC marker) in cultured bmDC from mice. B) ELISA for IFN- $\gamma$  of splenocytes, derived from MUC1-T immunized MUC1 TG mice, stimulated *in vitro* by bone marrow derived DC pulsed with MUC1-T glycoprotein (N=4).

Based on the fact that antigens from MUC1 glycoprotein carrying the T antigen appear to be capable of being taken up and presented to T lymphocytes by bmDC in mice, we investigated binding of MUC1-T to bmDC. Moreover, as the MUC1-Tn and MUC1-STn (MUC1-STn currently under investigation in our laboratory, unpublished data) glycoforms have been shown to bind calcium dependent type lectins such as the macrophage galactose C-type lectin (MGL) (Napoletano C. *et al.* 2007) on human monocyte derived DCs, MUC1-T calcium dependent binding to bmDC was investigated. MUC1-T was biotinylated and coupled to FITC microbeads (see materials and methods) prior to incubation with bmDC in the presence or absence of calcium. As illustrated in figure 4.7, MUC1 glycoprotein carrying the T antigen binds to immature bone marrow derived DC in a calcium dependent manner, and that this particular binding can be abolished by pre-incubating the bmDC with Gal $\beta$ 1-3GalNAc bound to polyacrylamide agarose (PAA).



*Fig 4.7: MUC1-T binding to murine DC*

Flow cytometer analysis of microbead coupled MUC1-T binding to bone marrow derived immature DCs.

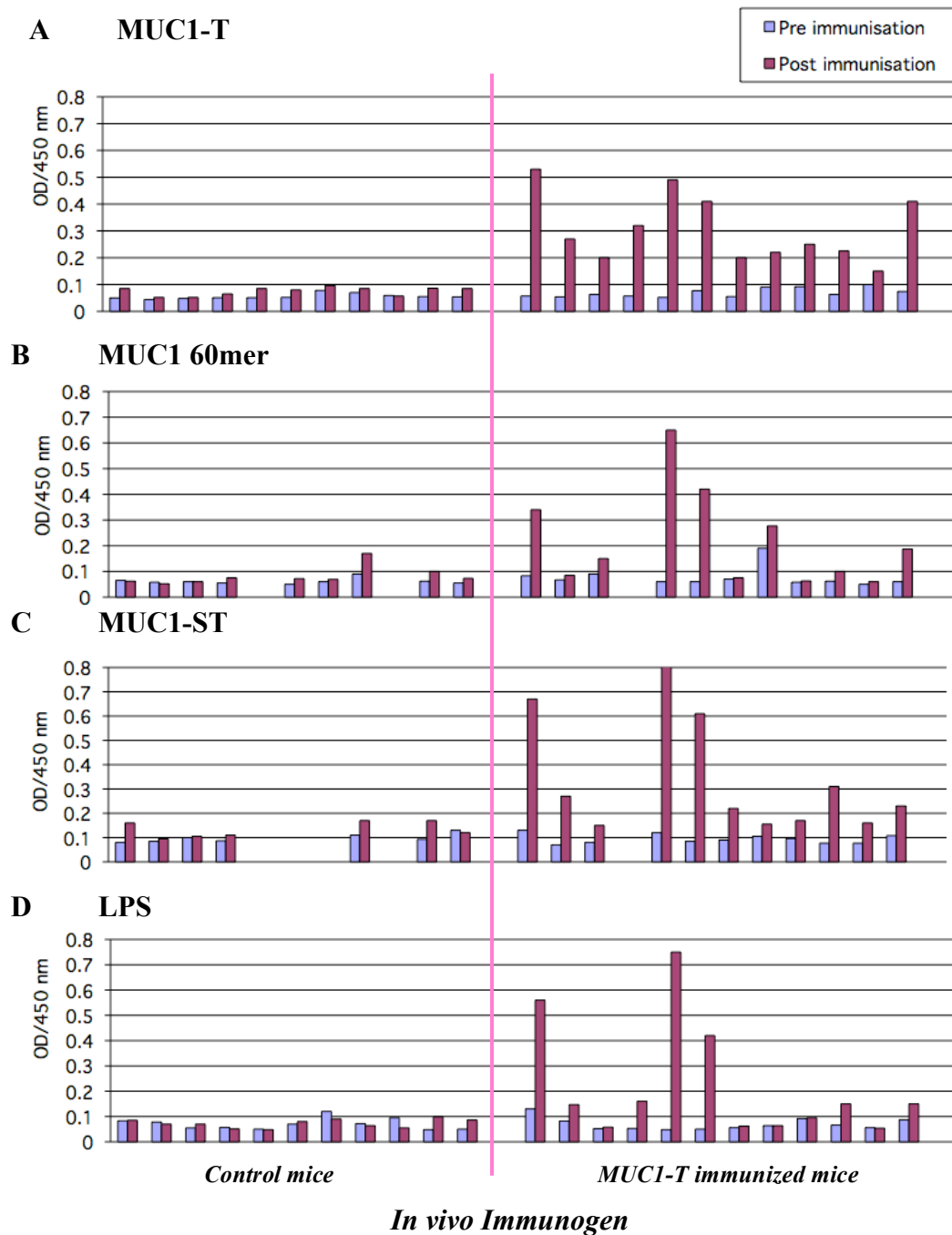
#### **4.2.4 Humoral responses to MUC1-T glycoprotein**

In cancer, O-linked changes in glycosylation of MUC1 affects the profile of epitopes presented to B cells, and this concept is one of the factors that instigated interest in MUC1 as an immunotherapeutic target. Although, the role of the humoral response in tumour immunology is by no means clear, it is established that antibody levels to MUC1 in breast cancer patients can correlate with metastatic free survival and improved overall survival (von Mensdorff-Pouilly S. *et al.* 1996, Blixt O. *et al.* 2011). Moreover, as a small increase in cytokines unique to the Th2 type of response was observed in mice immunized with 10 µg of MUC1-T, the humoral response represented by the production of antibodies to MUC1 was investigated.

Figure 4.8 shows the results from an ELISA for MUC1 glycoforms using serum samples from MUC1 transgenic mice taken pre and post immunization with PBS and adjuvant (Freund's) or MUC1-T plus adjuvant. The immunization schedule consisted of 10 µg of the immunogen plus adjuvant given three times three weeks apart (see materials and methods). The results indicate that 11 out of 12 tested sera derived from the post-immunized mice contain antibodies to the immunogen MUC1-T (fig 4.8A). In addition a number of mice appear to have produced antibodies able to recognize MUC1 expressing a different glycostructure from the one of the immunizing immunogen. Moreover, antibodies have been found to recognize MUC1 core protein, which was tested by using a 60mer peptide consisting of three tandems repeats (fig 4.8B), and to MUC1 carrying sialyl T (Neu5Ac- $\alpha$ 2,3Gal $\beta$ 1,3GalNAc) (fig 4.8C).

Although all our glycoproteins and peptides used in our immunization and *in vitro* studies contained extremely low endotoxin levels (>1 EU/ml). Sera from mice immunized with MUC1-T or control (PBS + adjuvant) were tested for antibodies to LPS. As seen in figure 4.8D, four out of twelve mice immunized with MUC1-T appear to have generated antibodies, which bind LPS, while no antibodies to LPS were generated in the control mice.





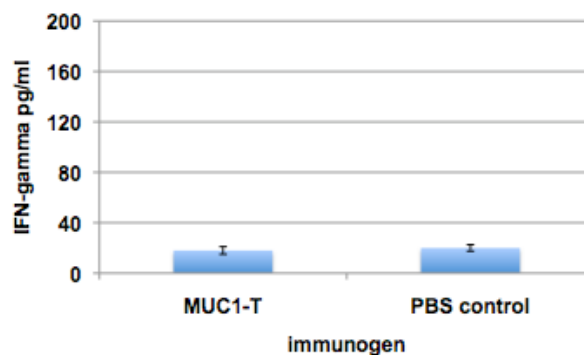
**Fig 4.8:** *Humoral responses in MUC1 TG mice immunized with MUC1-T glycoprotein*

Antibody levels in sera (1:40 dilution) from mice pre and post immunization with PBS + adjuvant (n=11), or MUC1-T + adjuvant (n=12) were assayed in an ELISA against: **A**, 50 ng/well of purified MUC1-T. **B**, 50 ng/well of 60mer MUC1 peptide. **C**, 50 ng/well of purified MUC1-ST. **D**, 50 ng/well of LPS.

Note: blank spaces due to non-availability of serum for testing.

#### **4.2.5 Lack of response to MUC1-T in MUC1 Tg mice living in SPF environment.**

Due to high incidence of bacterial and viral pathogens, within the animal unit housing the MUC1 Tg mice, the facility underwent a re-derivation program of all the mice to become a specific pathogen free (SPF) facility. Following the re-derivation program, the response induced by the immunization in MUC1 Tg mice, was lost (fig 4.9). Unfortunately from this time only MUC1 Tg mice housed in the SPF facility were available.



*Fig 4.9: Cellular response to MUC1-T glycoprotein was lost when the MUC1 Tg mice were re-derived into SPF facility.*

Single cell suspension of splenocytes from mice immunized with MUC1-T glycoprotein or PBS control (10 µg per injection x 3) were incubated with MUC1-T glycoprotein (10 µg/ml) for 52 hours. The supernatant was then analysed for IFN-γ production by ELISA. The results shown are the average of 8 mice per group.

The disappearance of the cellular response in MUC1 Tg to MUC1-T has been a major setback, as further experiments were not possible. However, the testing of MUC1 based immunogens on the MUC1 transgenic mice living in a non SPF environment indicates that under particular conditions MUC1 tolerance can be broken. The lack of response of the clean mice is almost certainly due to the environment affecting their immunological predisposition. Mice living in a less clean environment would have a more educated immune system. For example the mice living in the “dirty” area were carriers of *Helicobacter pylori* (*H. pylori*), a bacterium that resides in the stomach, which has been linked to the increase in antibodies to MUC1 (Klamas K. *et al.* 2007) and T antigen (Klamas K. *et al.* 2002). Thus speculatively, in MUC1 Tg mice living in a dirty environment the recombinant

MUC1-T used as an immunogen may be able to induce an immune responses with help from other cellular components of the immune system primed by common antigenic structures such as the T antigen.

#### 4.2.6 Response to MUC1 sialyl T

Vaccination of MUC1 transgenic mice, living in a “dirty” environment, with MUC1 carrying sialyl T (Neu5Ac- $\alpha$ 2,3Gal $\beta$ 1,3GalNAc) failed to induce an immune response (fig. 4.1). Furthermore, MUC1-ST could not induce cellular or humoral immune responses even in wild-type mice where the immunogen was foreign (fig 4.10, and table 4.1). However, in wild-type mice a fusion protein of MUC1-ST and mouse Fc could induce a humoral response that was directed to MUC1-ST (table 4.1) indicating that the common sialyl-T glycan (present on both glycoproteins) was not masking immunogenic epitopes. These data are in keeping with the mounting evidence that MUC1 carrying sialylated-T may have immunosuppressive properties (Mungul A. *et al.* 2004, Monti P. *et al.* 2004, Rughetti A. *et al.* 2005).

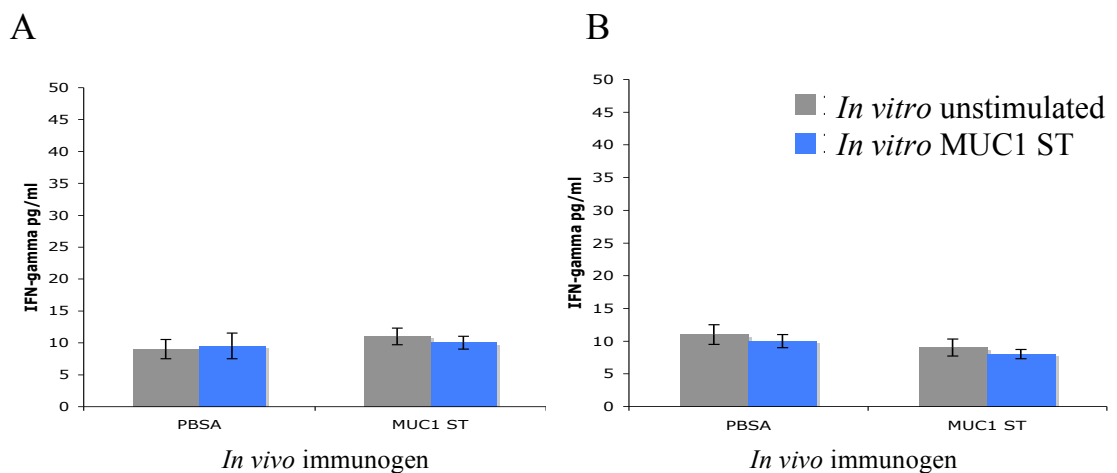


Fig 4.10: Cellular response to MUC1 carrying sialyl T in WT mice

ELISA for IFN- $\gamma$  from splenocytes stimulated *in vitro* by 10  $\mu$ g/ml of MUC1 carrying the Sialyl-T glycan (Neu5Ac $\alpha$ 2,3Gal $\beta$ 1,3GalNAc), following the *in vivo* priming (10  $\mu$ g x 3) with MUC1-ST, in A: Balb/c (n=6) B: C57Bl/6 (n=12) WT mice.

Table 4.1: Humoral response to MUC1 carrying sialyl-T in WT mice.

Table representing mAb responses to MUC1 peptide (60 mer), recombinant MUC1-ST and MUC1-T by ELISA on sera (dilution 1/40) +ve = 3 fold or more Optical Density increase from pre-immunization control.

<i>Immunogen</i>	<i>Sera tested on MUC1-T</i>	<i>Sera tested on MUC1-ST</i>	<i>Sera tested on MUC1 60 mer</i>
MUC1 ST-IgG	6/7 +ve	10/10 +ve	4/7 +ve
<b>MUC1 ST</b>	<b>7/7 -ve</b>	<b>10/10 -ve</b>	<b>7/7 -ve</b>
PBS	10/10 -ve	10/10 -ve	10/10 -ve

### 4.3 Discussion

In this chapter it has been shown that MUC1-T can induce a cellular response, which is mainly Th1 in MUC1 transgenic mice. However, this immune response was lost when the mice were re-derived into a SPF environment. It has also been shown that MUC1-ST could not induce an immune response even in wild-type mice where the protein is foreign. This was not due to the non-immunogenic sialyl-T glycan masking the protein core as when the MUC1-ST-Ig fusion protein was used as an immunogen an immune response could be generated.

As mentioned in the introduction of this chapter, the over-expression and aberrant O-linked glycosylation of MUC1 make it an attractive immunotherapeutic target for the treatment of breast and other cancers. Thus, having MUC1 expressed in large amounts on the entire cell surface and having the formation and exposure of novel glycan epitopes should, without external intervention, prime the immune system. Indeed early breast cancer patients have been found to have naturally occurring anti-MUC1 antibodies in their sera, which are reactive with tumour-associated glycoforms, and these patients have a decreased incidence of breast cancer metastasis and an increased time to progression (von Mensdorff-Pouilly S. *et al.* 2000a, Blixt O. *et al.* 2011).

As described in chapter 1, the abnormal glycosylation of MUC1 in breast cancer results in the exposure of tumour specific Tn (GalNAc), and sialyl-Tn (Neu5Ac $\alpha$ 2-6GalNAc), and tumour-associated T (Gal $\beta$ 1-3GalNAc) and sialyl-T (Neu5Ac $\alpha$ 2,3Gal $\beta$ 1,3GalNAc) glycans. Thus, MUC1 expressing these glycans is of particular interest as a target for immunotherapeutic treatment of cancer. Indeed,

studies with MUC1-based immunogens, using glycopeptides from the TR of MUC1, have found that immunization with MUC1-Tn and MUC1-STn glycopeptides elicits strong humoral responses even in MUC1 Tg mice (Sorensen A. L. *et al.* 2006). A clinical trial which used STn linked to KLH (Keyhole limpet hemocyanin), named Theratope®, as a vaccine was started a few years ago. This vaccine reached phase III, but failed to demonstrate a significant effect versus the control vaccine (Holmberg L. A. and Sandmaier M. A. 2004). However, the trial failed to include as an entry requirement that patients' tumours should express sialyl-Tn, and as only 25-30% of breast cancers express this glycan the possibility of benefit in a subset of patients whose tumours expressed sialyl-Tn could have been missed. Moreover, in a mouse model the sialyl-Tn linked to KLH formulation (Theratope®) was found to successfully promote antibody-mediated inhibition of tumour growth by targeting multiple sialyl-Tn-carrying proteins, not just MUC1, and crucially this inhibition required sialyl-Tn expression by the tumour (Julien S. *et al.* 2007).

Testing of MUC1 glycoproteins carrying either Tn, T, sialyl-T or no O-linked glycans found that of these only MUC1 carrying the glycan T was able to break cellular tolerance in MUC1 Tg mice and induce a cellular response measured by IFN- $\gamma$  and IL-2 secretion. IFN- $\gamma$  is the main cytokine secreted by the Th1 subset of T lymphocytes expressing CD8 (cytotoxic T cells) or CD4 (helper T cells). Depletion of CD4 and/or CD8 T cell subsets showed that the cells expressing CD4 were the source of the IFN- $\gamma$ . Furthermore, we demonstrated that dendritic cells are able to present peptide from MUC1-T glycoproteins to primed CD4 +ve T lymphocytes *in vitro*. Binding studies showed that MUC1-T can bind to murine bone marrow-derived DC in a calcium dependent manner, suggesting a C-type lectin interaction that may involve mMGL-2.

In the human system MUC1 glycoproteins carrying GalNAc have been shown to bind to MGL on immature DC, and it has been speculated that MGL-MUC1-Tn interactions could be involved in the maintaining the localization of immature DC at strategic sites. This is of particular importance as MGL appears to mediate DC migratory capability (Van Vliet S. J. *et al.* 2008). Furthermore, it has also been demonstrated that MGL functions as an antigen uptake receptor that facilitates

antigen presentation by the MHC (Napoletano C. *et al.* 2012). In the mouse system, two C-type macrophage galactose-type lectins (mMGL1 and mMGL2) appear to be orthologs of the human MGL, however the preferential carbohydrate specificity is different, with mMGL2 preferentially binding  $\alpha$ - and  $\beta$ -GalNAc conjugated soluble polyacrylamides, whereas mMGL1 preferentially binds Lewis X conjugated soluble polyacrylamides. However, mMGL1 could also bind to the T glycan on polyacrylamide (Tsuiji M. *et al.* 2002). The ability of the glycan Gal $\beta$ 1-3GalNAc to block binding of MUC1-T to immature murine dendritic cells confirms that the binding is dependent on the glycan and suggests that MGL1 may be the lectin involved. This could be confirmed by the use of blocking antibodies to MGL. This could explain how MUC1-T is taken up by mouse immature DC and possibly macrophages, and epitopes presented to CD4<sup>+</sup> T cell.

Interestingly, the response in MUC1 TG mice brought about by the immunization with MUC1-T was not seen in mice derived and bred in a specific pathogen free environment, thus suggesting that the environment affected their immunological predisposition and this is discussed further in Chapter 6. Mice living in a less clean environment have been shown to have a more educated immune system (Makinodan T. *et al.* 1971). Moreover, the MUC1 transgenic mice in the “dirty” area were carriers of *Helicobacter pylori* (*H. pylori*) (determined during health screening), a bacterium that resides in the stomach, which has been linked to the increase in antibodies to MUC1 (Klams K. *et al.* 2007) and T antigen (Klams K. *et al.* 2002). Thus, possibly *H. pylori* bacterial infection may have contributed by epitope spreading to an increased immune response to MUC1-T cellular responses.

MUC1-ST immunisation did not elicit immunological responses even in wild-type mice where it is a foreign antigen. However, it was found that by immunising wild-type mice with the fusion protein of MUC1-ST and the Fc region of mouse Ig2a, a humoral response directed to MUC1-ST was induced. The fact that the fusion protein MUC1-ST-Ig elicited a MUC1-specific humoral response suggests that this is routed through a different pathway in the cells and may be taken up through binding to Fc receptors (Revetch J. V. and Bolland S. 2001). However the lack of immunological responses in wild-type mice after immunization with MUC1-ST without the Ig,

suggests that this glycoprotein may have immunosuppressive properties. Indeed, secreted cancer-associated MUC1, which is also likely to contain MUC1-ST has been associated with immunosuppression through inhibition of T-cell proliferation and maturation of dendritic cells (Agrawal B. *et al.* 1998, Rughetti A. *et al.* 2005). Moreover, sugars associated with aberrant glycosylated MUC1, have been suggested to be responsible for the observed anti-proliferative effects on cancer cell lines, such as the T47D (Paul S. *et al.* 1999). In addition to this, recent data generated Dr Beatson in our lab indicate that recombinant MUC1-ST binds to several sialic-acid-binding immunoglobulin-like lectins (Siglec). The vast majority of Siglecs have been found on immune cells, including DC, macrophages, T cells, B cells and NK cells, mediating a wide range of activities including cell death (Jandus C. *et al.* 2011). Inhibitory signals can be mediated through the ITIMs present in the cytoplasmic tails of the majority of Siglecs (Avril T. *et al.* 2006, Crocker P. R. *et al.* 2007).

At the present time, evidence is mounting on the possible immunosuppressive role of the MUC1-ST glycoform that is commonly found on MUC1 in breast cancer. Although the ST glycoform is not itself tumour-associated, it is not normally carried on epithelial mucins as in the tumour situation which results in this glycan being repeated hundreds of time on a single molecule. This may allow the strong interaction with inhibitory lectins.

The results in this chapter have shown that only a specific glycoform of MUC1, MUC1-T, can induce a cellular immune response, whereas the MUC1-ST glycoform may be immunosuppressive or at the very least be unable to induce immunity. The results are important for any studies on the use of the MUC1 glycoprotein in immunotherapy and could have far reaching implications for the efficacy of immunological responses to cancer and perhaps immunological responses in general. The sialyltransferase responsible for the expression of sialyl-T is ST3Gal-I which is over-expressed in breast cancer. In the next chapter the effect of over-expression of this enzyme in the mammary gland on the development of spontaneous mammary tumours is investigated.

## ***Chapter 5***

***Development of human ST3Gal-I transgenic mice and the involvement of ST3Gal-I in tumorigenesis***



## 5.1 Introduction

The previous results chapter has covered how aberrant glycosylation of MUC1 can induce an immune response as seen with MUC1-T, in MUC1 transgenic mice. However the MUC1-ST glycoform was unable to induce an immune response even in wild-type mice where this antigen was foreign. Moreover, these data and data from other groups (Monti P. *et al.* 2004, Ruggetti A. *et al.* 2005) suggest that MUC1-ST may be immunosuppressive. The glycosyltransferase responsible for the formation of ST is ST3Gal-I, thus in this chapter the influence of over-expressing ST3Gal-I on tumorigenicity *in vivo* was examined.

The major mechanism in breast cancer that results in aberrant O-linked glycosylation is changes in the expression of glycosyltransferases. In breast cancer, the sialyltransferase ST3Gal-I is over-expressed compared with normal mammary epithelial cells (Burchell J. *et al.* 1999). This sialyltransferase adds sialic acid to the galactose residue of core 1 (Gal $\beta$ 1,3GalNAc) O-glycans, resulting in sialyl-T being found in many breast cancers. The ability of up-regulated ST3Gal-I to sialylate core 1, even in the presence of the competing enzyme C2GnT1 has been demonstrated *in vitro* by transfection experiments (Dalziel M. *et al.* 2001) as described in the introduction chapter. To further understand the relation between ST3Gal-I overexpression and breast cancer, this chapter describes the development of transgenic mice that overexpress human ST3Gal-I in epithelial cells, including those of the mammary gland. This was achieved by driving expression of the ST3Gal-I transgene from the human MUC1 promoter and resulted in ST3Gal-I expression in tissues where MUC1 is found in humans. Once these mice had been characterized, the effect of overexpressing this sialyltransferase was investigated by crossing them with mice that spontaneously develop mammary tumours (PyMT mice).

## 5.2 Results

### 5.2.1 Generation of the human ST3Gal-I transgenic mouse

To enable expression of human ST3Gal-I in the mammary gland and its upregulation during pregnancy, lactation and in mammary tumours, the translated region of hST3Gal-I (+889 to +1951, GenBank L29555) was placed under the control of the MUC1 promoter (see materials and methods for construct details). The region of the MUC1 promoter used was -1401 to +33 which is 74% homologous with the mouse Muc1 promoter (Spicer A. P. *et al.* 1991), and has been shown to be sufficient to direct tissue-specific expression in murine models, allowing up-regulation during pregnancy and lactation and in PyMT inducible mammary tumours (Graham R. A. *et al.* 2001). This region of the MUC1 promoter and the ST3Gal-I cDNA was cloned into the vector pNASS $\beta$  (see materials and methods) and the ST3Gal-I together with the MUC1 promoter was isolated from the vector by cutting the section with the restriction enzymes Sal I and Sca I (fig 5.1), before running it into 0.7% agarose gel and purifying the insert using a gel extraction kit from Qiagen (see materials and methods). DNA was dissolved in injection buffer (0.1 mM ethylenediaminetetraacetic acid, 10 mM Tris pH 7.4 prepared in ultrapure water), and 5ng were injected into the nucleus of pure C57/Bl6 mouse embryos. These embryos were subsequently transferred into day 1-plugged pseudopregnant C57Bl/6 female mice (injections carried out by the CR-UK transgenic service).

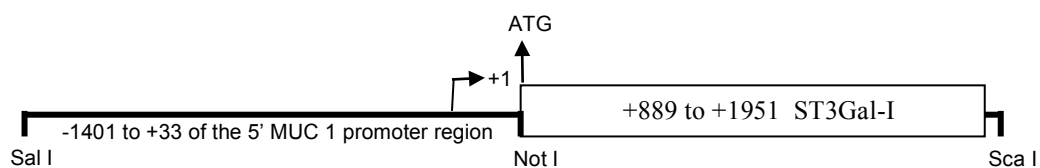


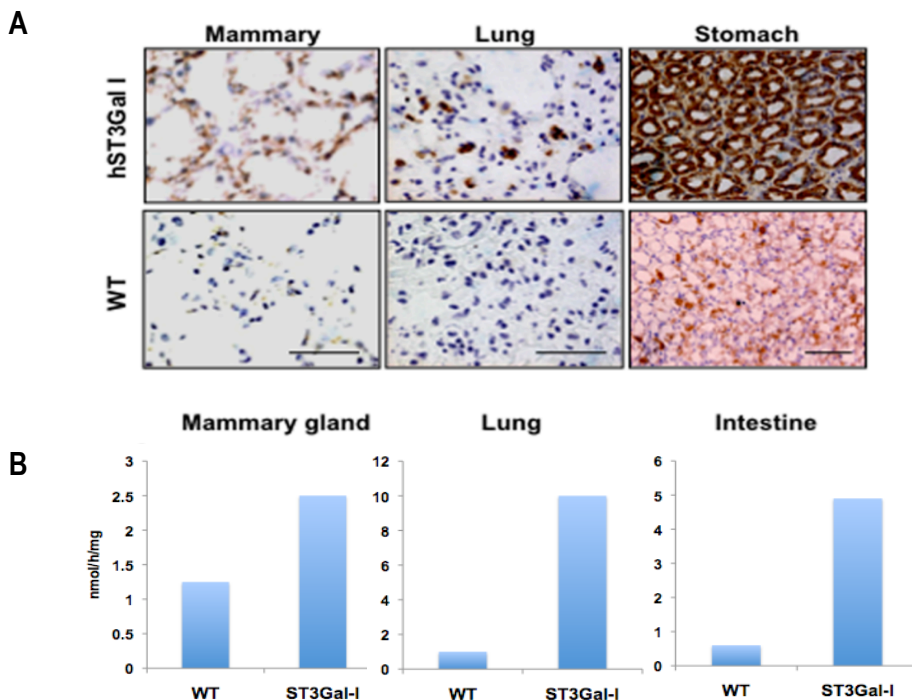
Figure 5.1: Diagrammatic representation of hST3Gal-I DNA construct used for the generation of ST3Gal-I transgenic mice

Transgenic mice were identified by PCR on DNA isolated from tail snips, amplifying a 550 base pair segment of DNA using oligonucleotide primers within the 3' end of the MUC1 promoter region and the 5' end of the ST3Gal-I region (see materials and methods for details). Two founder mice were identified, but only one expressed ST3Gal-I in the expected tissues. Further expansion of the line was

developed by littermate mating, and homozygosity confirmed by backcrossing mice onto WT C57Bl/6.

### 5.2.2 Characterisation of *hST3Gal-I* transgenic mice

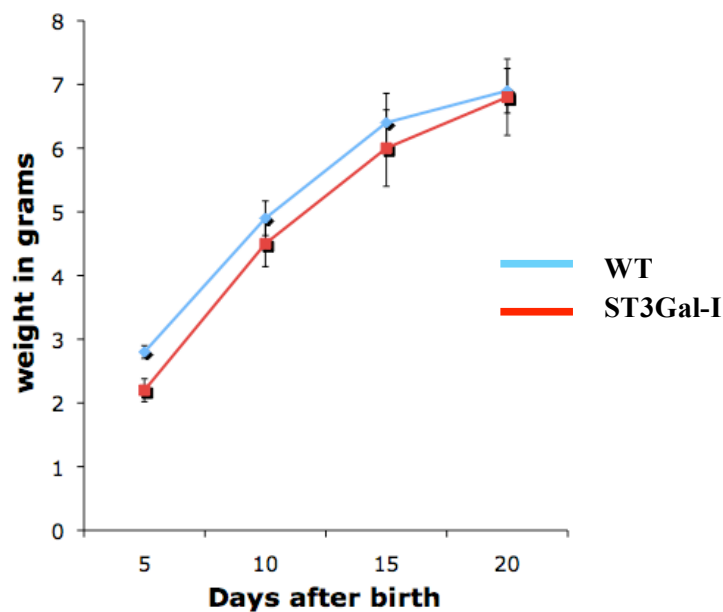
Expression of hST3Gal-I was determined by immunohistochemistry using the monoclonal antibody 4B10, which specifically reacts with human ST3Gal-I and not with the mouse enzyme (Vallejo-Ruiz V. *et al.* 2001). As expected from the activity of the MUC1 promoter (Graham R. A. *et al.* 2001), the hST3Gal-I enzyme was found expressed in the epithelial cells of tissues where Muc1 is found (Peat N. *et al.* 1992), such as mammary glands, stomach, lungs (fig 5.2A), pancreas and salivary glands. To ensure that the expressed human ST3Gal-I was enzymatically active, ST3Gal activity was measured in the mammary glands, lungs and intestine in collaboration with Dr Inka Brockhausen. As shown in figure 5.2B, the ST3Gal activity, which includes human and mouse using this assay, was found to be higher in ST3Gal-I Tg mice as compared to wild-type controls.



**Figure 5.2:** Human ST3Gal-I is expressed in the correct tissues and is active in ST3Gal-I transgenic mice

*A:* Immunohistochemical staining (frozen sections) of tissue from the hST3Gal-I transgenic mouse strain and C57Bl/6 WT control, using anti human ST3Gal-I antibody. Scale bar represents 100  $\mu$ m.  
*B:* Enzymatic ST3Gal activity of tissue from the hST3Gal-I transgenic mice and C57Bl/6, using Gal $\beta$ 1-3GalNAc $\alpha$ -pnp as the acceptor substrate (performed by Dr Inka Brockhausen).

Homozygous hST3Gal-I Tg mice developed normally and had no overt abnormal physical or behavioural characteristics, but initially appeared to be smaller at birth. Thus, in order to assess whether the mice grow at the normal rate after birth, the weight of pups were recorded and compared to wild-type C57Bl/6 mice. The individual weights from four litters of mice derived from the hST3Gal-I Tg and four litters from the WT C57Bl/6 mice were compared (fig 5.3). At days 5 and 10 the weight of the majority of the hST3Gal-I pups was less, as compared to the control group, and these differences were found to be statistically significant by unpaired T test, with respective p values of  $>0.001$  and  $0.0246$ . However, by day 15 and just before weaning at day 20, there was no difference in the weight of the hST3Gal-I Tg pups compared to the control group. As no gross differences in the mammary gland was observed (see below), it is possible that the close-relative breeding could be the cause for the reduced birth weight in the hST3Gal-I mice. However, a defect in milk composition cannot be ruled out.



*Figure 5.3: Weight of hST3Gal-I pups*

*Weight in grams of pups from hST3Gal-I homozygous parents and WT C57Bl/6 mice parents taken individually at day 5, 10, 15 and 20 after birth. Results represent the mean  $\pm$  SD of 4 litters (20 pups WT, and 19 pups hST3Gal-I) derived from two breeding pairs per group.*

### 5.2.3 Mammary gland development in pregnant and lactating ST3Gal-I mice

Muc1 is highly expressed in mammary epithelial cells during pregnancy and lactation (Schroeder J. A. *et al.* 2001). As the expression of the human ST3Gal-I is being controlled by the MUC1 promoter, it was of interest to investigate any morphological differences in breast tissue of the hST3Gal-I Tg mice. At a gross visual examination, the shape and size of the mammary glands of hST3Gal-I, their littermate (fig. 5.4 B) and wild-type C57Bl/6 mice (data not shown) was found to be similar.

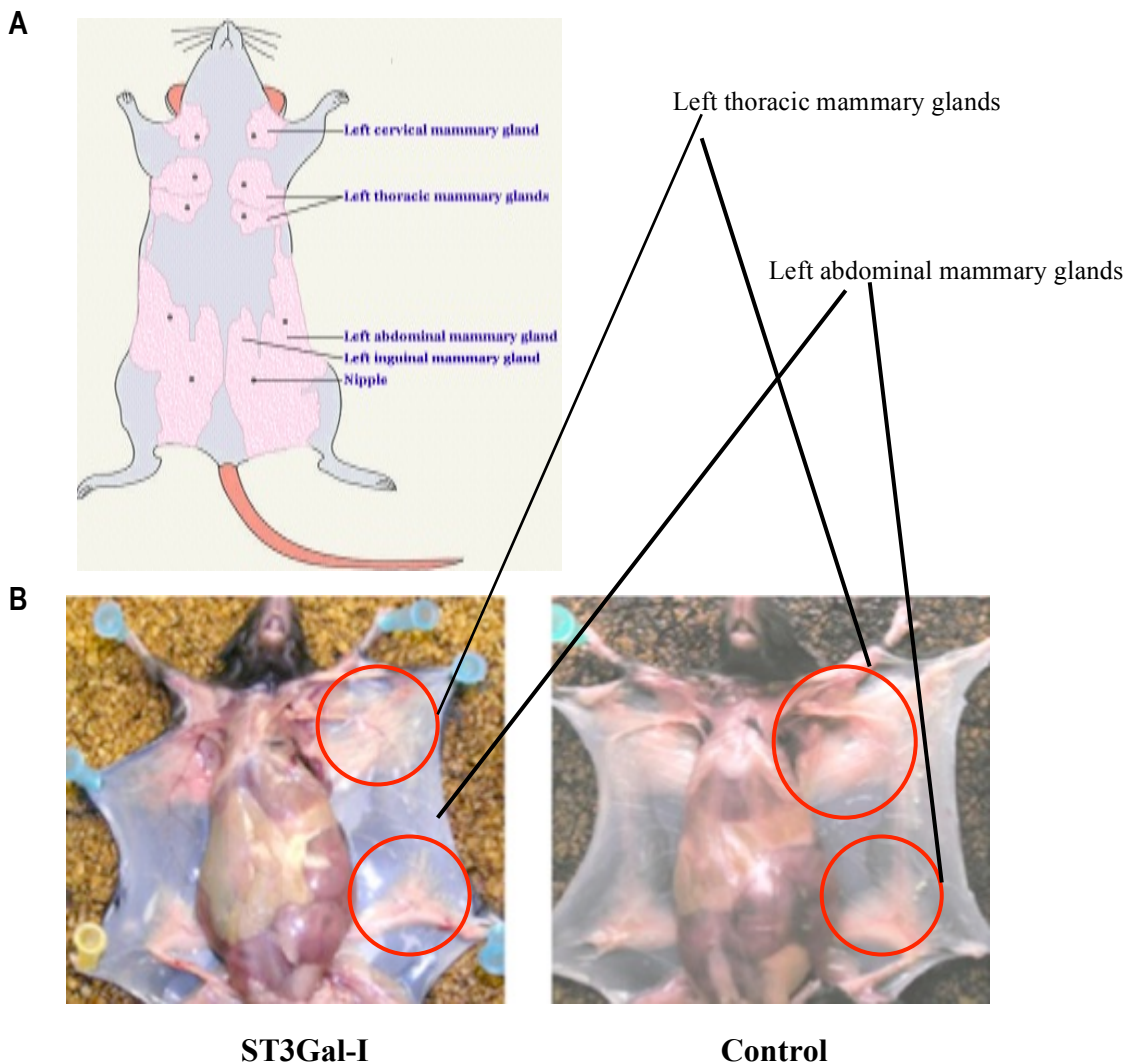
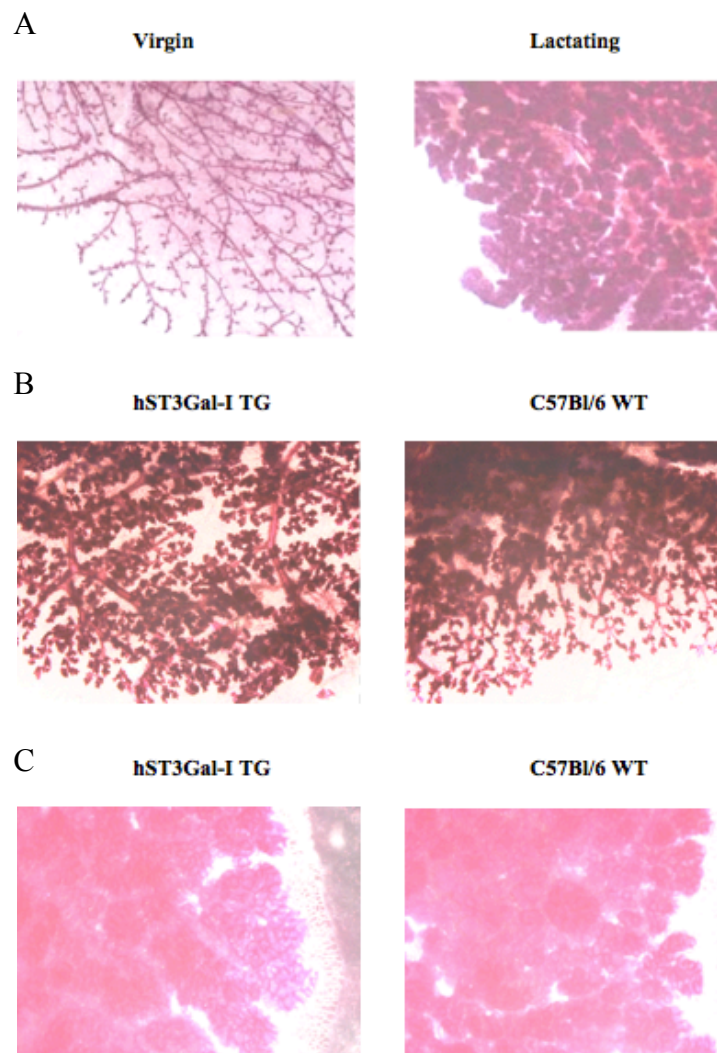


Figure 5.4: Mammary gland appearance in mice

A; Diagram of mammary gland (www. Atlas of laboratory mouse histology). B; Examples of sub epidermal gross appearance of mammary glands from the hST3Gal-I Tg mice and C57Bl/6 WT littermate.

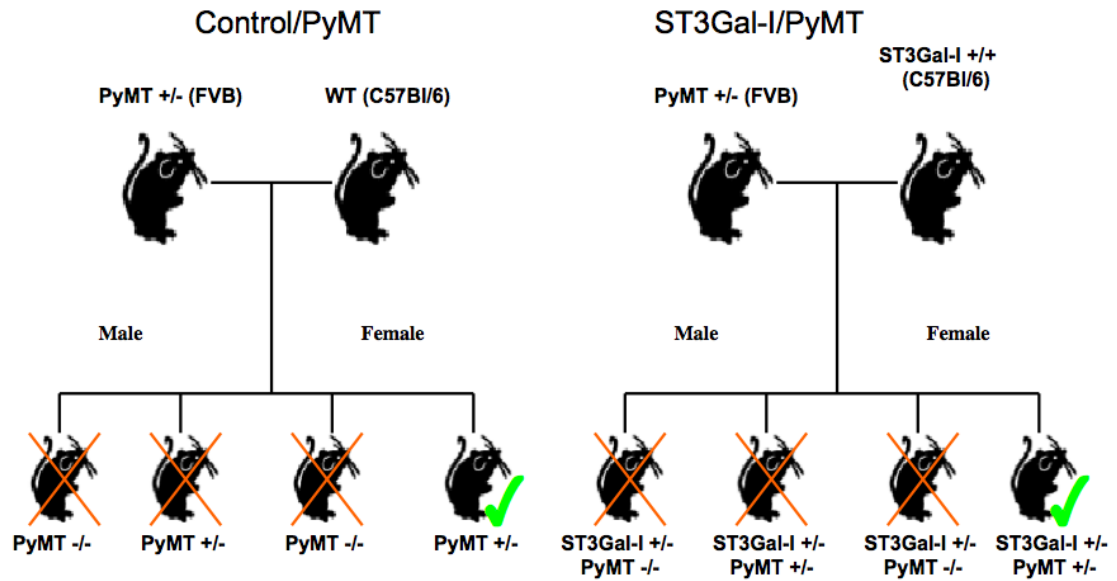
In order to assess any possible microscopic morphological changes, mammary glands from the hST3Gal-I mice and the control mice were carmine stained (fig. 5.5). Mammary glands at day 16 of pregnancy, and day 10 of lactation were chosen for assessment as at 16 days of pregnancy the mammary gland is actively proliferating and by day 10 of lactation the majority of the rapid proliferation of the mammary epithelium would have occurred (fig. 5.5B and 5.5C). Comparison of the carmine staining did not reveal major differences in numbers or branching of alveoli between the two groups. However, to definitively show that increased expression and activity of ST3Gal-I did not alter the morphology of the mammary gland, a more detailed investigation, which would involve taking glands at different days during development, pregnancy, lactation and involution needs to be undertaken.



**Figure 5.5:** Carmine staining of mammary glands from hST3Gal-I TG mice  
*A:* Example of carmine staining of mouse mammary glands from a virgin and a lactating mouse. *B* and *C:* Example of carmine staining of mouse mammary glands from hST3Gal-I and C57Bl/6 mice, at *B* day16 of pregnancy and *C* at day 10 of lactation.

#### **5.2.4 Development of spontaneous mammary tumours over-expressing ST3Gal-I**

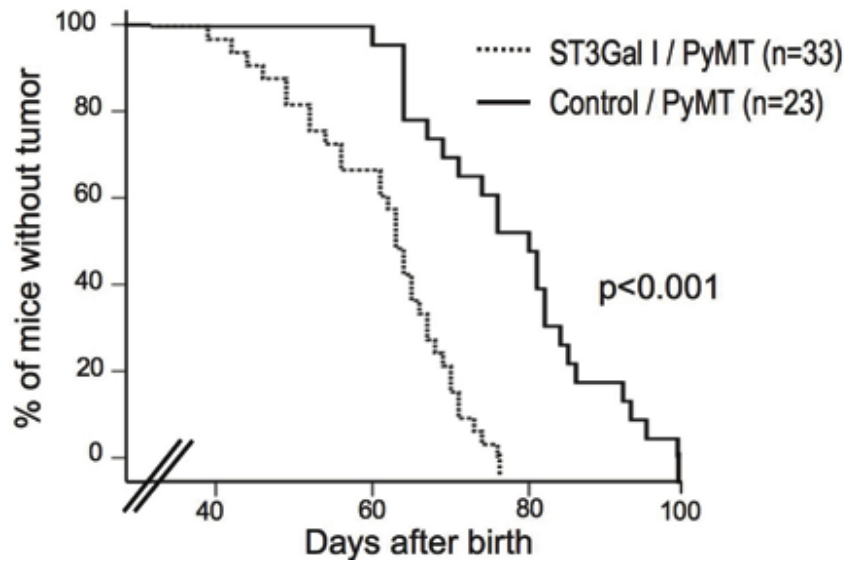
To evaluate the impact of increased ST3Gal-I enzyme levels on mammary tumour formation, ST3Gal-I Tg mice were crossed with mice carrying the polyoma middle T antigen (PyMT mice). Since their generation, PyMT mice have been well established as a murine model resembling human breast cancer (Gendler S. J. and Mukherjee P. 2001). PyMT mice carry the middle T oncogene under the transcriptional control of the mouse mammary tumour virus promoter/enhancer which results in widespread transformation of the mammary epithelium with rapid growth of multi-focal mammary adenocarcinomas (Guy C. T. *et al.* 1992). The original investigation by Chanteale Guy *et al.* in 1992 used FVB mice and found that 100% of the mice expressing the promoter/enhancer developed adenocarcinomas and all the tumours become detectable by palpation between the ages of 3 to 6 weeks. However, differences in tumour development were found in different founder mice, and it was suggested that these differences might be the result of different sites of gene integration. When PyMT FVB mice were crossed with C57Bl/6 mice or crossed with C57Bl/6 mice homozygous for the *MUC1* gene, 100% of the mice developed mammary adenocarcinomas, similarly to that described by Guy and colleagues. However the time for the tumours to be detectable by palpation was found to be between 8 to 15 weeks of age (Chen D. *et al.* 2003, Mukherjee P. *et al.* 2007). Thus, as the appearance of tumours in different stains of mice have substantial timing differences, the importance of using mice of an identical strain as controls when measuring the development of tumours was paramount, and figure 5.6 shows our strategy.



*Figure 5.6: Schematic representation of crossing of ST3Gal-I with PyMT mice and WT (C57Bl/6) with PyMT mice*

In investigating the kinetics of tumour initiation in PyMT control (C57Bl/6 x PyMT) and ST3Gal-I/PyMT mice, the percentage of palpable tumour-free mice per total number of mice observed over time was calculated. Between the PyMT control (n=23) and ST3Gal-I/PyMT (n=33) we observed a clear significant difference ( $p < 0.001$ ), in the time to tumour development (fig. 5.7). In the control PyMT mice, the mammary tumours started to appear from day 60 and by day 100 all of the mice had at least one palpable tumour. Within two weeks of the appearance of the first palpable tumour, all of the females went on to develop multifocal tumours in the remaining glands. In contrast, in the ST3Gal-I/PyMT mice tumours started to appear significantly earlier at day 38, and by day 77 all of the mice had developed palpable mammary tumours, also becoming multifocal within two weeks of the appearance of the first palpable tumour.



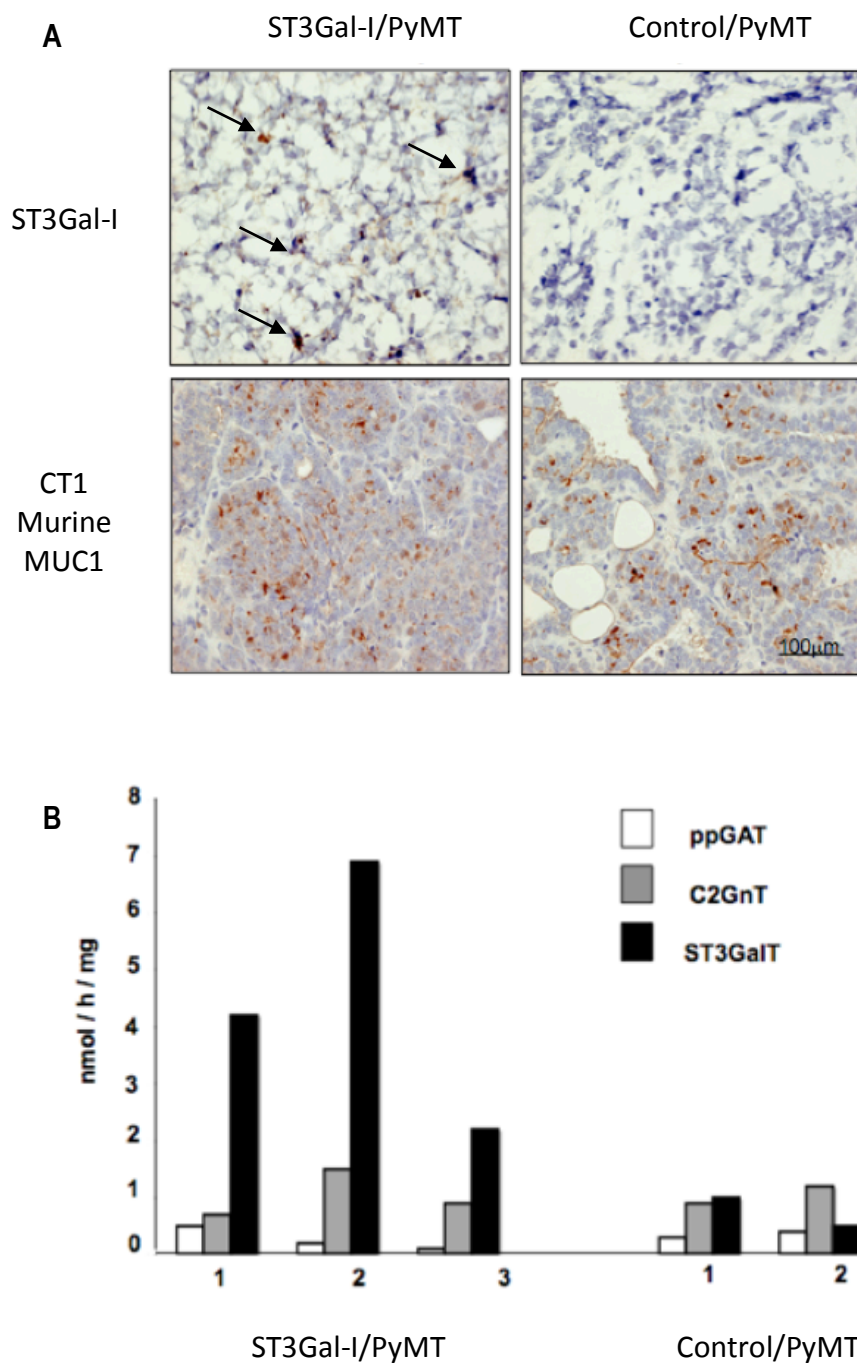


**Figure 5.7:** *ST3Gal-I/PyMT mice develop tumours faster than control mice on the same background*

*Kinetics of tumour formation in female F1 control/PyMT (C57Bl6/PyMT +/- n=23) (—), and F1 ST3Gal-I/PyMT (hST3Gal-I+/-/PyMT+/- n=33) (----). Percentage of mice without tumours was calculated as the number of tumour-free mice compared with the number of mice observed for the initiation of a palpable tumour. The difference between the curves was ( $p<0.001$ ) evaluated by the Breslow test, where a  $p$  value of less than 0.1 is considered significant.*

### **5.2.5 Expression of hST3Gal-I and Muc1 in PyMT mammary tumours**

Expression of human ST3Gal-I and murine Muc1 in three formalin fixed, paraffin embedded mammary tumours was analysed by immunohistochemistry (see materials and methods), using a human monoclonal antibody for human ST3Gal-I (4B10) (Vallejo-Ruiz V. *et al.* 2001) and a polyclonal antibody to the human MUC1 cytoplasmic tail that also reacts with mouse Muc1 cytoplasmic tail (CT1) (Pemberton L. *et al.* 1992). Human ST3Gal-I sialyltransferase was found to be express only in tumours derived from the ST3Gal-I/PyMT Tg mice, while the expression of Muc1 was found to be similar in tumours from the transgenic and control mice (fig 5.8A). In addition, ST3Gal enzyme activity (carried out by Dr Inka Brockhausen) was increased in tumours arising from the ST3Gal-I/PyMT Tg mice, as compared to tumours arising from the controls PyMT mice (fig 5.8B).



**Figure 5.8:** *hST3Gal-I and murine Muc1 expression in mammary tumours.*

*A: Staining for ST3Gal-I (frozen sections) and Muc1 (formalin fixed paraffin embedded sections) of tumours from C57Bl6/PyMT +/- (control/PyMT) and hST3Gal-I+/-/PyMT+/- (ST3Gal-I/PyMT) mice. Scale bars represent 100µm and arrows point to strong area of staining.*

*B: Enzymatic expression in tumours from the transgenic mouse strain ST3Gal-I/PyMT and control/PyMT, using Galβ1-3GalNAcα-pnp acceptor substrate.*

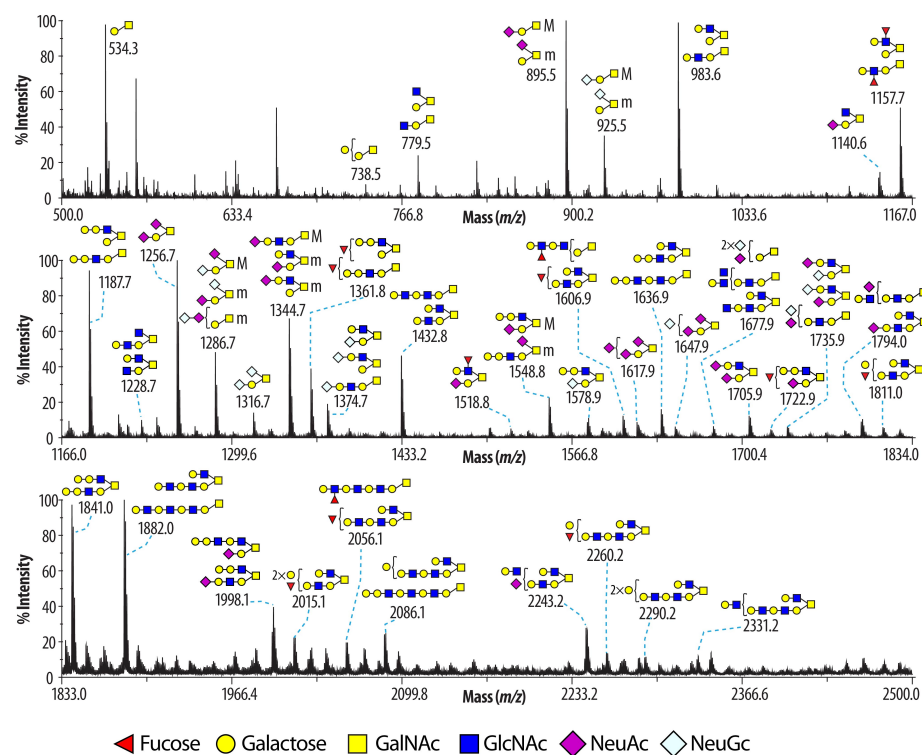
The activities of two other families of glycosyltransferases were also measured. The polypeptide GalNAc transferases that are responsible for adding GalNAc to serine

and/or threonine and C2Gn transferase that add GlcNAc to core 1 (see section 1.3.4, table 1.3 and figure 1.7). As can be seen from figure 5.8B, no differences in activity of these glycosyltransferases were observed from tumours samples arising from ST3Gal-I/PyMT Tg mice, as compared to tumours samples arising from the controls PyMT mice.

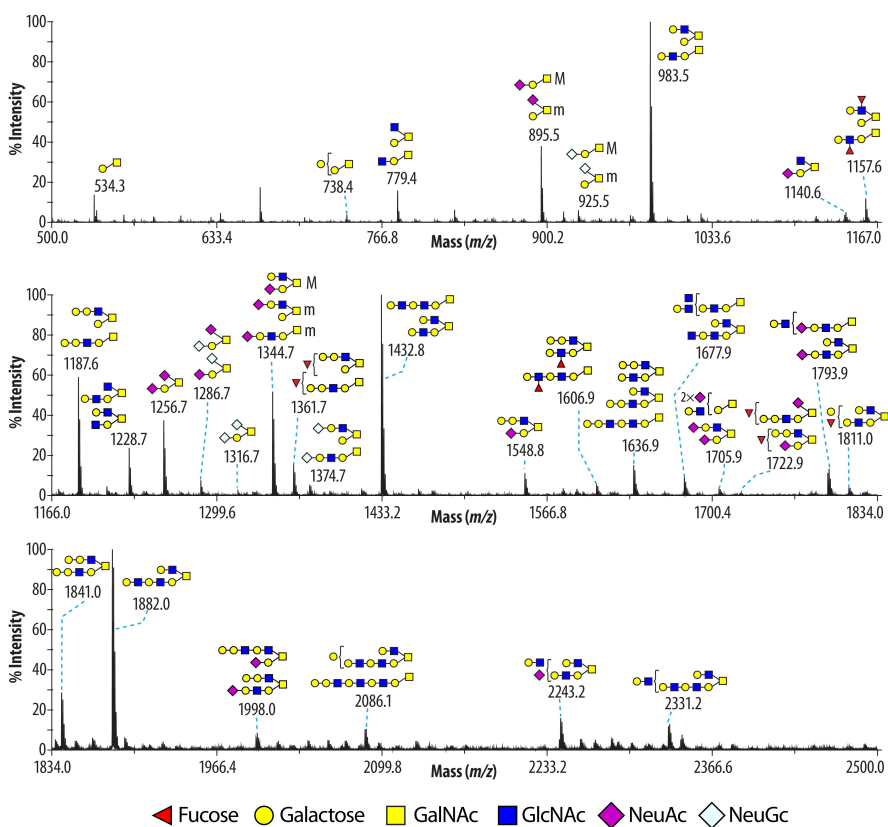
### **5.2.6 Mass spectrometry *O*-glycans profiles of hST3Gal-I tumours**

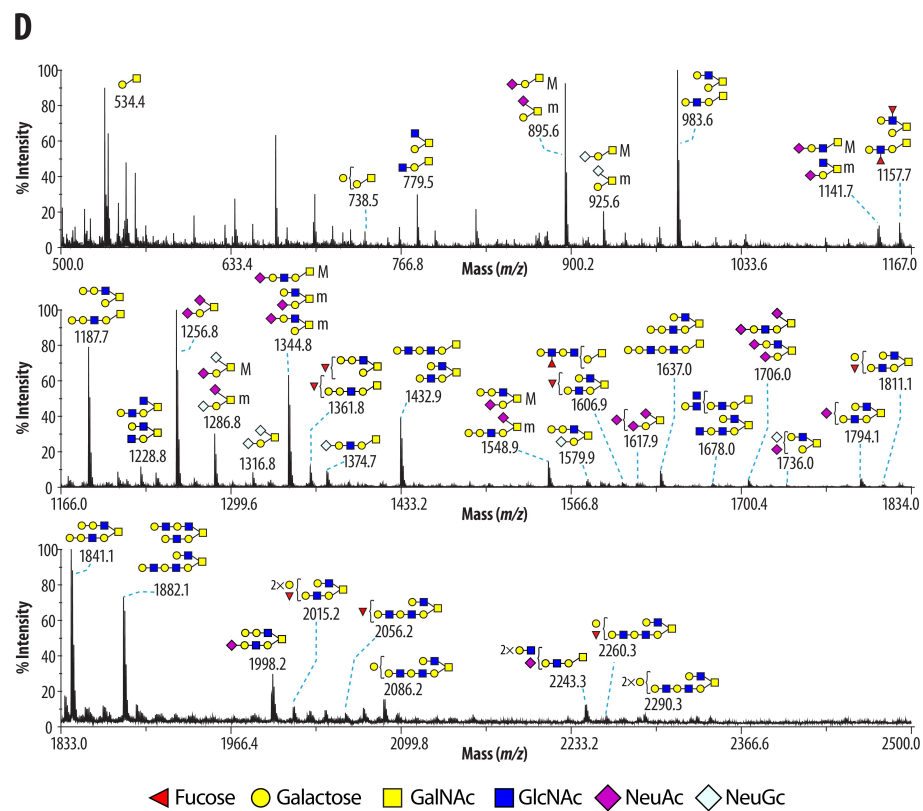
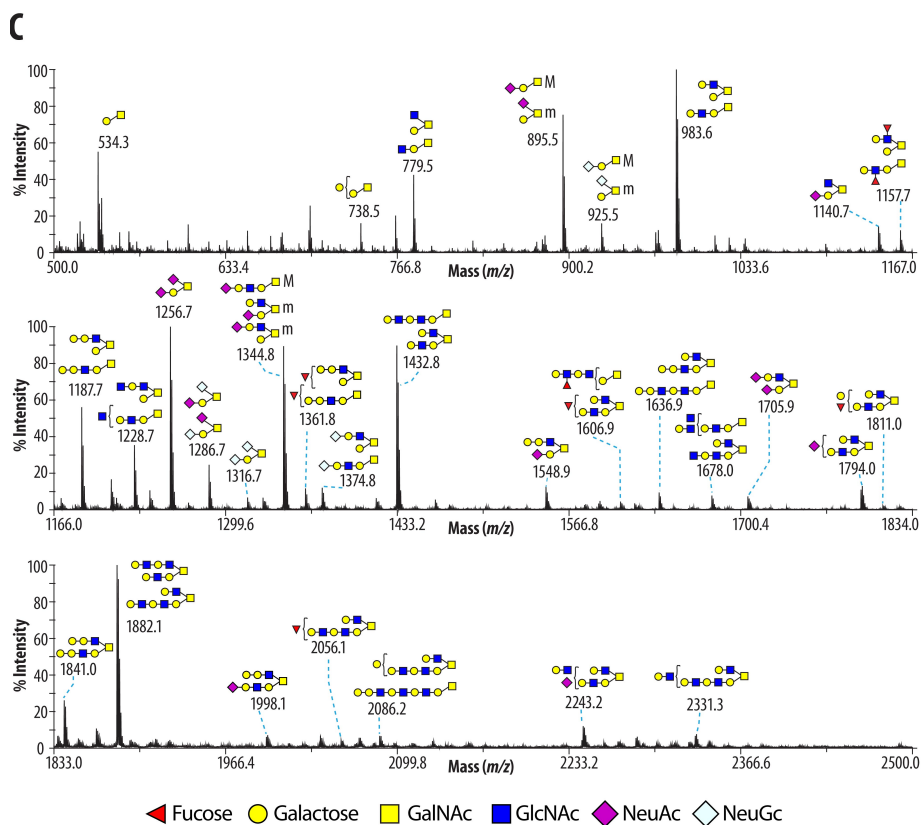
In order to investigate possible differences in the *O*-glycan profiles of ST3Gal-I tumours and those arising in the controls, two tumours derived from hST3Gal-I/PyMT mice and three tumours derived from the control/PyMT mice were analysed by mass spectrometry by the group of Professor Ann Dell (Imperial College) (fig 5.9). It is notable that the *O*-glycan profiles are very variable, irrespective of the group's provenience, with the exception of one of the tumours derived from the control group that had a low level of the core 1 type of *O*-glycans which included T, and ST (fig 5.9B). However, to further explore this type of variability an increased number of tumour samples would need to be tested, which are not available. As murine mammary tumours show changes in O-linked glycosylation with increased core 1 glycans (Graham R. A. *et al.* 2001), it is possible that in these end point tumours, over-expressing ST3Gal-I does not have any additional effect.

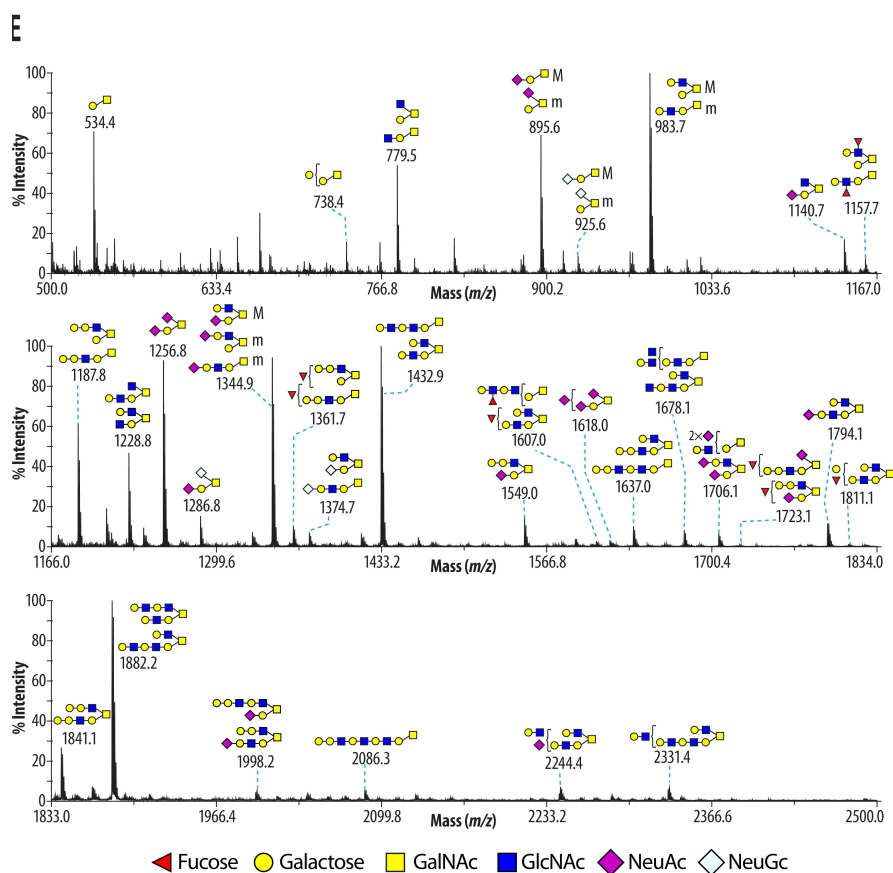
**A**



**B**







**Figure 5.9:** MALDI-TOF spectra of permethylated O-glycans derived from control/PyMT and ST3Gal-I/PyMT mice tumours.

A, B and C O-glycan profiles are from control/PyMT mice tumours; D and E O-glycan profiles are from ST3Gal-I/PyMT mice tumours. Profiles of O-glycans are from the 35% MeCN fraction from a  $C_{18}$  Sep-Pak. All molecular ions are  $[M+Na]^+$ . Putative structures based on composition, tandem mass spectrometry and the literature are shown. Cartoon structures are according to the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>) guidelines. Structures that show sugars outside a bracket have not been unequivocally defined. Performed by Professor Anne Dell's group at Imperial College London.

### **5.2.7 Histology of tumours**

Forty-two tumours developed by 33 hST3Gal-I/PyMT mice and 33 tumours developed by 22 control C57/PyMT mice were used to assess the microscopic anatomy. The tumours were fixed, paraffin embedded and H&E stained as described in the materials and methods, before being assessed blindly by Professor Sarah Pinder (Breast Pathologist). Tumour grade is a powerful indicator of prognosis and the three main characteristics used in grading human breast carcinomas, are glandular differentiation, nuclear pleomorphism and mitotic count. This grading system was used to assess the tumours from the mice (Table 5.1, 5.2 and 5.3). Each element is given a score of 1 to 3. The lowest possible score ( $1+1+1=3$ ) is given to well define tumours that form tubules and have a low mitotic rate measured by high power field (HPF) of  $<10/10$  HPF. The highest possible score is 9 ( $3+3+3=9$ ), and the score of all three components (glandular differentiation, nuclear pleomorphism and mitotic count) are added together to give the "grade" ( $3-5=$  grade 1,  $6-7=$  grade 2 and  $8-9=$  grade 3) (Bloom H. J. and Richardson W. W. 1957). According to the above grading system called Scarff-Bloom-Richardson grading system, the majority of the tumours developed by hST3Gal-I/PyMT or by the control C57/PyMT mice have a similar grade (grade 2).

In addition to the characteristics used to grade the tumours, the degree of apoptosis (Table 5.4), necrosis and heterogeneity was estimated (Table 5.5). The results indicate that the tumours developed by hST3Gal-I/PyMT or by the control C57/PyMT mice were similar, with no major morphological differences. There was a small trend for the ST3Gal-I tumours to have a smaller % of cells undergoing apoptosis. However, this was only a slight trend ( $P=0.0983$  unpaired T test) and so it was concluded this is likely to fall within the normal variable range.

Score	C57xPyMT	ST3Gal-IxPyMT
1	-	-
2	89%	98%
3	11%	2%

*Table 5.1: Glandular score*

Glandular % score, equivalent of human breast cancer glandular score (score 1 = >75%, score 2 = 10-75%, score 3 = <10% )

Score	C57xPyMT	ST3Gal-IxPyMT
1	3%	2%
2	73%	75%
3	24%	23%

*Table 5.2: Pleomorphism Score*

Pleomorphism score, equivalent of human breast cancer pleomorphism score: 1= mild (little difference from normal), 2= moderate, 3= large (pleomorphic cancer cells)

Score	C57xPyMT	ST3Gal-IxPyMT
1	12%	14%
2	21%	19%
3	67%	67%

*Table 5.3: Mitotic count per 10 HPF*

Numerical mitotic score, equivalent of human breast cancer mitotic score: 1=>10, 2= 10 to 20, and 3 =<20

Category	C57xPyMT	ST3Gal-IxPyMT
Mild	24%	37%
Moderate	45%	37%
Marked	31%	26%

*Table 5.4: Estimation of the degree of apoptosis*

Estimate % of cells undergoing apoptosis (Categories: mild, moderate, marked)

	C57xPyMT	ST3Gal-IxPyMT
A: Heterogeneity	72%	74%
B: Necrosis	45%	38%

*Table 5.5: Estimation of the degree of heterogeneity and necrosis*

% of tumours with: A, heterogeneity (presence of different cytological appearances within the tumour). B, necrosis



### **5.2.8 TGF- $\beta$ in serum of ST3Gal-I/PyMT mice**

Expression of sialylated core 1 glycans on MUC1 results in increased tumour growth in a transplantable tumour model in immuno-competent mice, but not in immuno-compromised mice, suggesting an immunological mechanism (Mungul A. *et al.* 2004). As it is known that neoplastic cells themselves, including tumours cells from PyMT mice generate immunosuppressive molecules, including transforming growth factor- $\beta$  (TGF- $\beta$ ) (Fischer J. R. *et al.* 1994), and that TGF- $\beta$  produced by many cancers results in increased regulatory T cells (Treg), immuno-incompetent immune cells and poor prognosis (Flavell R. A. *et al.* 2010) it was decided to investigate the presence of TGF- $\beta$  in sera from the mice. Thus, sera from female mice, Control/PyMT and ST3Gal-I/PyMT, were taken before the appearance of the palpable tumours (20 to 40 days old), and the level of TGF- $\beta$  tested by ELISA.

TGF- $\beta$  levels in the sera from ST3Gal-I/PyMT mice were found to be higher as compared with the levels in the sera from the control/PyMT mice ( $P < 0.001$  unpaired t test) of similar age (fig. 5.10A). However, as TGF- $\beta$  levels in serum increased with tumour size (fig. 5.10B), it seems likely that the higher level of TGF- $\beta$  found in sera from the ST3Gal-I/PyMT mice is a consequence of the early development of the tumours. Moreover, no different was observed in TGF- $\beta$  expression when analyzed by qRT-PCR using RNA derived from the Control/PyMT control and ST3Gal-I/PyMT tumours (fig. 5.10C). Thus again indicating that the differences in serum level of TGF- $\beta$  are reflecting the increased tumour size of the ST3Gal-I/PyMT tumours, confirming that the ST3Gal-I/PyMT mice develop tumours faster than the controls.

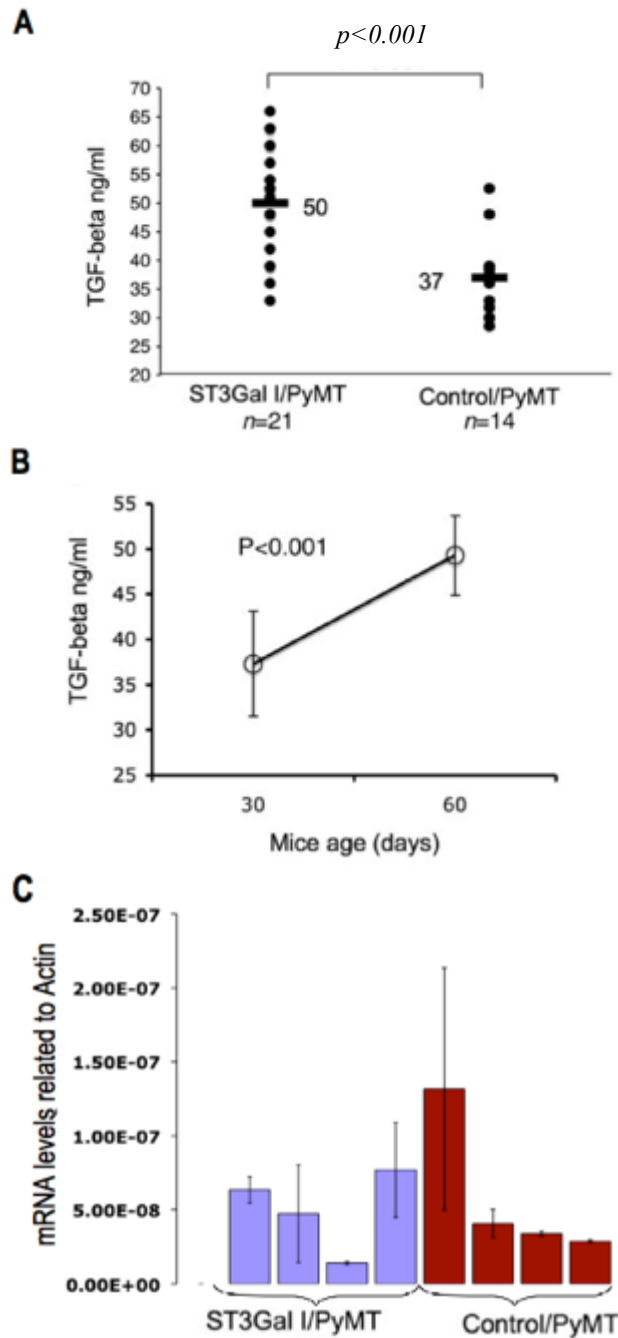


Figure 5.10: ST3Gal-I/PyMT mice have increased TGF- $\beta$  in their serum which reflect increased size of their tumours

A) Sera from Control/PyMT (C57Bl6/PyMT +/-  $n=14$ ), and ST3Gal-I/PyMT (hST3Gal-I +/- /PyMT +/-  $n=21$ ) mice, taken between the age of 20 to 40 days, and analysed for TGF- $\beta$  by ELISA. The difference between groups was evaluated by two-tailed T test ( $p < 0.001$ ). B) Sera from PyMT control (C57Bl6/PyMT +/-  $n=6$ ) mice, taken at 30 and 60 days of age, analysed for TGF- $\beta$  by ELISA. The difference between the two time points was evaluated by two-tailed T test ( $p < 0.001$ ). C) Level of mRNA expression for TGF- $\beta$  measured by real time polymerase chain reaction and represented as fold change relative to actin mRNA level. RNA was derived from cell lysate of 4 tumours from Control/PyMT (C57Bl6/PyMT), and ST3Gal-I/PyMT (hST3Gal-I +/- /PyMT +/-) mice.

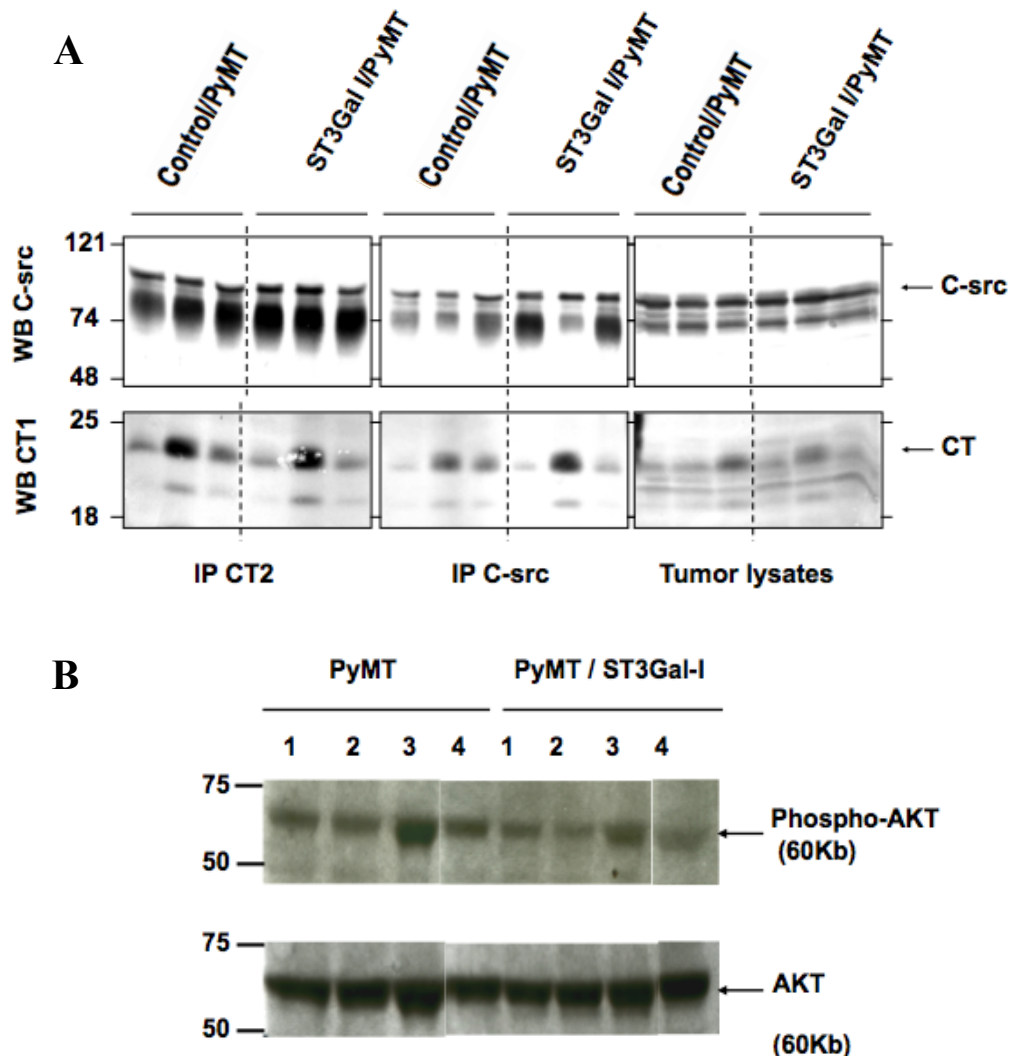
### **5.2.9 MUC1 signalling in hST3Gal-I/PyMT tumours**

As discussed in Chapter 1 the interaction of MUC1 cytoplasmic tail with a variety of proteins has long been recognized (Singh P. K. and Hollingsworth M. A. 2006). For example MUC1-CT interacts with EGFR, c-Src, and  $\beta$ -catenin, which are involved in neoplasia and cell-cell interaction (Pandey P. *et al.* 1995, Yamamoto M. *et al.* 1997, Li Y. *et al.* 2001, Ren J. *et al.* 2002). c-Src activation is essential for the induction of mammary tumours in the PyMT Tg mice and murine Muc1 has been demonstrated to interact with c-Src in PyMT induced mammary tumours (Al Masri A. and Gendler S. J. 2005). Moreover, in Muc1 null mice the development of PyMT induced tumours is significantly delayed (Spicer A. P. *et al.* 1991)

As Muc1 carries so many O-linked glycans that may be affected by the over-expression of ST3Gal-I, we investigated whether the expression of hST3Gal-I in PyMT tumours alters the interaction between Muc1 and cSrc, leading to the observed early appearance of the tumours. The tumour cell lysates from three hST3Gal-I/PyMT or control/PyMT were used to immunoprecipitate Muc1 and c-Src with either a monoclonal antibody to the cytoplasmic tail of Muc1 (CT2) or with an anti-c-Src antibody (Molecular Probes) (see figure 5.11A). Indeed, c-Src Western blot staining of Muc1 immunoprecipitated tumour samples (fig 5.11A upper panels), and Muc1 staining of c-Src immunoprecipitated tumours samples (fig 5.11A lower panels), confirm the previously reported (Al Masri A. and Gendler S. J. 2005) interactions of Muc1 with c-Src. However, no consistent difference between tumours derived from the two groups of mice was observed.

PI3-kinase is also a direct target of PyMT oncogenesis and phosphorylates AKT, which is itself a protein kinase. We therefore investigated the phosphorylation of AKT. Figure 5.11B shows four tumours cell lysates derived from hST3Gal-I/PyMT or control/PyMT mice that were used to assess the levels of AKT and its phosphorylation. In figure 5.11B lower panel it is possible to see that the levels of AKT, evaluated using a AKT specific monoclonal Ab (Cell Signaling 9272), which runs at about 60 kDa in a SDS gel, are similar. Figure 5.11B upper panel, shows the reaction with a monoclonal antibody to phosho AKT (Cell Signaling 4058S).

However, there appears to be no consistent differences in the phosphorylation of AKT between tumours derived from the transgenic or wild-type mice.



*Figure 5.11: Increased ST3Gal activity does not affect the interaction of Muc1 with c-Src or down-stream signalling.*

*A; Tumour lysates (1 mg) from control/PyMT (n=3), and ST3Gal-I/PyMT (n=3) mice were immunoprecipitated with anti c-Src or anti Muc1 (CT2) and immunoblotted for c-Src (upper panels) or Muc1 (CT1) (lower panels). B; Tumour lysates (30 µg) from control/PyMT mice (n=4), and ST3Gal-I/PyMT mice (n=4) were immunoblotted for phosphorylated-AKT (upper panel), or total AKT (lower panel).*

### 5.3 Discussion

*In vitro* studies have shown that over-expression of ST3Gal-I is sufficient to cause the change from core 2 based glycans to sialyl core 1 glycans (sialyl-T) carried on mucins commonly found in breast cancer (Brockhausen I. 2006, Dalziel M. *et al.* 2001). However, it is not clear if the expression of the sialyl-T glycan by epithelial cancers has any direct role in driving tumorigenesis. To address this question we developed a transgenic mouse that expresses hST3Gal-I from the MUC1 promoter to induce this sialyltransferase expression in epithelial tissues including the mammary gland and mammary tumours (Graham R. A. *et al.* 2001). These mice were crossed with MMTV-PyMT mice that develop spontaneous mammary tumours (Guy C. T. *et al.* 1992) and the progeny developed tumours significantly faster than the controls. This is the first report that shows that an enzyme involved in O-linked glycosylation promotes tumorigenesis.

Mice transgenic for the human ST3Gal-I sialyltransferase were generated by pronuclear injections into early C57/Bl6 mouse embryos (see materials and methods), a well-established technique that results in insertion of the chosen gene into the genome. Due to the nature of this technique, the hST3Gal-I could have been inserted at a single or multiple sites into the genome. However, from 1000 injected embryos only two out of 40 mice born expressed the transgene, thus the likelihood of multiple insertions is believed to be low. Southern analysis can be performed at this stage to assess if the transgene is intact and if the transgene has integrated into multiple sites, however, tissue expression and enzyme activity were chosen to assess the expression and the integrity of the transgene. The mice expressing the hST3Gal-I were found to express the sialyltransferase in tissues where Muc1 is expressed, such as stomach, lungs, liver, intestine, salivary glands and the mammary glands, in accordance with the data from Graham and colleagues (Graham R. A. *et al.* 2001). This confirms that -1401 to +33 of the MUC1 promoter has all the elements required for expression in these tissues. Furthermore, the glycosyltransferase was found to be active in tissues such as the intestine, lungs and mammary glands. Although the enzyme assay cannot distinguish between enzymes from different species, the increased activity observed in the ST3Gal-I Tg mice indicates that a proportion of

this is human ST3Gal-I. The generated hST3Gal-I transgenic mice had no overt abnormal physical or behavioural characteristic.

Altered ST3Gal-I sialyltransferase expression in organs such as the mammary glands could have affected their lobuloalveolar development, or even the biosynthesis of milk produce. Thus, particular attention was paid to the growth of the pups from the hST3Gal-I Tg mice. Indeed pups derived from hST3Gal-I mice were found to weigh less soon after birth and up to day 10 of age as compared to mice of the same background, however this difference was small and the weight was recovered by day 20. Moreover, no overt differences in mammary gland morphology between the hST3Gal-I and control mice were observed.

In order to better understand ST3Gal-I involvement in oncogenesis, the hST3Gal-I transgenic mice were crossed with MMTV-PyMT mice that develop spontaneous mammary tumours (Guy C. T. *et al.* 1992). In this *in vivo* context, which has been designed to be as close as possible to human breast cancer development, we have been able to assess the effect of the overexpression of ST3Gal-I on tumour development. The data presented here clearly show that ST3Gal-I promotes mammary gland carcinogenesis, as tumours arose significantly earlier in hST3Gal-I/PyMT mice than in control mice.

Increased ST3Gal-I activity was detected in the ST3Gal-I/PyMT tumours. However despite this, the glycosylation pattern of these tumours cannot be distinguished from the control/PyMT tumours, as determined by mass spectrometry. This might indicate that changes of glycosylation induced by increased ST3Gal-I only affects a specific and restricted portion of the tumour glycoproteins, which would not be detected using this gross analysis. Alternatively, when the tumours have developed and are larger enough to necessitate culling of the mice, the effect of expression of the ST3Gal-I transgene may not be able to compound the effect of endogenous aberrant glycosylation (Graham R. A. *et al.* 2001).

Glycosylation of extracellular domains can affect signalling and interactions of intracellular domains of a protein (Guo H. B. *et al.* 2007). We therefore looked at the interactions of murine Muc1, which carries many *O*-glycans and has been implicated

in the tumorigenesis of PyMT-induced tumours. Although the interaction with c-Src reported by others (Al Masri A. and Gendler S. J. 2005) was confirmed, no differences could be observed in tumours from the two sets of animals and no differences were observed in down-stream signalling. Moreover, when analysed at the endpoint of the experiment, the tumours derived from the hST3Gal-I/PyMT mice or control mice did not show significant differences in terms of morphology or mitotic index, suggesting that ST3Gal-I expression did not affect the histopathological type of the tumour.

Comparison of the levels of circulating TGF $\beta$  suggest that the difference in the development of the tumour had already occurred in the mice prior to the detection of palpable tumour masses. Taken together, our results indicate that ST3Gal-I exerts its influence early in tumour development. It is therefore important to analyse any differences at this early stage (see discussion in Chapter 6), perhaps by using tumour-imaging techniques. Several mechanisms could be responsible for promoting tumorigenesis, one of them being the ability of the tumour to escape the immune surveillance early in tumour development. Certainly, changes in mucin-type glycosylation can affect how MUC1 interacts with the immune system, and while the expression of some glycans may be active in stimulating an immune response (Napoletano C. *et al.* 2007, and this thesis), other glycans such as sialyl-T, may inhibit a response (Mungul A. *et al.* 2004, and this thesis), suggesting that the type of O-glycans expressed by cancer cells may be able to modulate the immune response to the tumour. Thus, investigating the early tumour development in the hST3Gal-I/PyMT mice has the potential of uncovering the mechanism by which ST3Gal-I can promote mammary gland carcinogenesis.

# ***Chapter 6***

***Summary, discussion & further investigations***



## **6.1 Production and purification of recombinant MUC1 glycoproteins**

Post-translational modifications are vital for the function, stability and *in vivo* half-life of many proteins. Thus, mammalian cell expression systems are the preferred method of choice in the biopharmaceutical industry for the production of therapeutic glycoproteins, and the most commonly used cell type is the Chinese hamster ovary (CHO) cell (Hossler P. *et al.* 2009, Li F. *et al.* 2010). CHO cells have proven over the years to be a reliable system to produce the quantity and quality of therapeutic agents required; The list of approved therapeutic agents by the European Medicine Agency that are produced in CHO cells is extensive and includes Herceptin (Trastuzumab) from Genentech and NeoRecormon from Roche.

As our understanding of the specific glycan profile of glycoproteins grows, so does our understanding of the effects of this specific glycosylation on their therapeutic efficacy. Glycans play a very important role in the therapeutic effectiveness of proteins and in determining their *in vivo* half-life (Elliott S. *et al.* 2003). Ideally, the production of individual proteins should be guided by a thorough understanding of their optimal glycan profile.

The glycosylation of specific glycoproteins relies on the glycosyltransferases expressed by the parent cell, therefore, by knowing the glycosyltransferase expression profile it is possible to predict, to some degree, the glycans carried on the proteins produced. For example, and in regard to the production of MUC1 glycoproteins, CHO cells do not express the glycosyltransferase core 2  $\beta$ 1,6-N-acetylglucosaminyltransferase-1 which is responsible for the formation of the core 2 structure (VanderElst I. E. and Datti A. 1998), therefore it was predicted that the type of O-linked glycosylation would be mainly core 1. This indeed turned out to be the case as the glycosylation on the MUC1 produced in wild-type CHO K1 cells was found to be mainly core 1 based with no core 2, which is similar to that previously reported for endogenous MUC1 from the breast carcinoma cell line T47D (Lloyds K. *et al.* 1996, Muller S. *et al.* 1999). Furthermore, in our hands the CHO K1 clone was optimal to produce recombinant MUC1 on a large scale, with further purification steps limiting the level of contaminants.

In order to produce recombinant MUC1 glycoproteins with another very specific tailored glycan profile, mutant CHO ldlD cells were also used in this study. The deficiency of UDP-Gal/UDP-GalNAc 4-epimerase present in the ldlD mutant CHO cells leaves them unable to synthesize galactose and GalNAc (Kingsley D. M. *et al.* 1986). However, as the wild-type glycosyltransferases are present the process can be reversed by providing the cells with exogenous sources of sugars, which are utilized via a salvage pathway that does not require participation of the 4-epimerase enzymes (Segal S. 1983). By culturing CHO ldlD cells transfected with the MUC1 (16TR)-IgG construct with or without an exogenous source of GalNAc, it has been possible to produce in large quantities recombinant MUC1 without O-linked glycosylation or recombinant MUC1 carrying only O-linked GalNAc. Thus the work reported in this thesis, the first of this kind, shows how mutant CHO ldlD cells can be used in the production of recombinant MUC1 glycoprotein without O-linked glycosylation or carrying only O-linked GalNAc.

## **6.2 *In vivo immune response to MUC1 glycoforms***

Over-expression and aberrant O-linked glycosylation of MUC1, coupled to the fact that loss of polarity of cancer cells results in MUC1 being found over the entire surface, make MUC1 an attractive immunotherapeutic target for the treatment of breast and other cancers. Moreover there is now evidence to suggest that MUC1 may play a role in driving tumorigenesis (Mungul A. *et al.* 2004, Ahmad R. *et al.* 2011, and this thesis). In a National Cancer Institute (NIH, USA) study to prioritize cancer antigens, MUC1 was ranked the 2<sup>nd</sup> (out of 75) most optimal antigens for vaccine development (Cheever M. A. *et al.* 2009), and its popularity as an antigen target is demonstrated by the numerous ongoing clinical trials (Beatson R. E. *et al.* 2010). It is well establish that cancer-associated changes in the glycans carried by MUC1 exposes predominantly the glycans Tn, Sialyl-Tn, T and Sialyl-T, however the role that each tumour associated glycan plays in tumorigenesis still remains elusive. In this thesis the immune response to specific MUC1 glycoforms has been explored to determine their potential for the immunotherapy of MUC1 expressing cancers.

In recent years work with glycopeptides *in vitro* has contributed substantially to the understanding of glycopeptide processing and presentation by APCs, and priming of T and B cells. Ninkovic and colleagues suggested in 2007 that in order to enable efficient processing by immunoproteasomes, MUC1 tandem repeat domain glycopeptides should fulfil some specific requirements when used in a synthetic vaccine formulation. These included: site specificity O-glycosylation (in red are the permissive sites: AHGVT**S**APD**T**RPAPGSTAPPA) which should only carry the O-glycan moieties GalNAc (Tn) or Gal $\beta$ 1-3GalNAc (T), and sialylated extension of these glycans should not be included as this reduces or blocks the processing by immunoproteasomes and prevents the glycosylated peptide fragment fitting into the binding groove of MHC class I proteins. Thus, immunization using densely glycosylated and sialylated MUC1 peptides is believed to be ineffective as a vaccine. However, this thesis demonstrates that MUC1-T with an average of 4.3 glycosylation sites per repeat could induce an immune response.

MUC1 tandem repeat peptides carrying GalNAc have been shown to bind antigen presenting cells such as DC and macrophages through MGL (Saeland E. *et al.* 2007, Napoletano C. *et al.* 2007), and that antigen presenting cells are capable of processing and presenting MUC1-Tn on both MHC class I and II (Napoletano C. *et al.* 2007, Lakshminarayanan V. *et al.* 2012). Moreover, it is possible, by using MUC1-Tn peptides, to generate MUC1-Tn specific CTLs in murine models ( Xu J. *et al.* 2004; Stepensky D. *et al.* 2006, Lakshminarayanan V. *et al.* 2012), and the peptide within the MUC1 repeat domain (SAPDT- $\alpha$ /GalNAc-RPAPG) was predicted to be able to bind MHC class I HLA-A 0201 (HLA-A2) molecules (Ninkovic T. *et al.* 2009). In the study conducted by Lakshminarayanan and colleagues, MUC1 peptides glycosylated with a single GalNAc (TSAPDT- $\alpha$ /GalNAc-RPAP) was linked to TLR2 agonist and a T helper peptide epitope (Lakshminarayanan V. *et al.* 2012). The resulting glycosylated MUC1 tripartite vaccine was found to be more effective than the unglycosylated MUC1 tripartite vaccine in generating therapeutic responses in MUC1 transgenic mice. The inclusion of a helper T cell epitope would increase the efficacy of this vaccine but it is also possible that optimal TLR2 stimulation is required. On the other hand, the TLR2 agonist may also instigate APC uptake allowing cross presentation.

In the present study the heavily glycosylated (average 4.3 sites out of a possible 5 per TR) MUC1-T recombinant glycoprotein elicited both Th1 and mild Th2 cellular responses in MUC1 Tg mice. Moreover, it was demonstrated that CD4 positive T cells were responsible for the response. T antigen expression has already been shown to be linked to poor prognosis in breast cancer patients (Wolf M. F. *et al.* 1988), and as described in the introduction, T glycans on MUC1 may play a role in metastasis (Yu L. G. *et al.* 2007). Interestingly, during the eighties and early nineties Dr. George Springer conducted a series of clinical studies in breast cancer patients using a vaccine composed of human, neuraminidase treated group O red blood cell membranes expressing predominantly the T antigen, with about 10% of the glycans being Tn antigen. The vaccine was administered with  $\text{Ca}_3(\text{PO}_4)_2$  and contained traces of *Salmonella typhi*, which also contains the T antigen, as the vaccine adjuvant. The patients were vaccinated over the course of years and this resulted in improved 5 and 10 years survival which was highly significant when compared to independent statistics of the United State National Cancer Institute (Springer G. F. 1997). Eventhough the Springer studies did not provide conclusive evidence on the efficacy of an anti-T antigen vaccine because of the design of the study and the use of historical controls, this early success, in my opinion should have led to further investigations. In a more recent study led by Professor Kate Rittenhouse-Olson, peptides that mimic the T antigen were used to vaccinate mice. The peptides were given with Alum adjuvant (aluminium hydroxide) and contained traces of inactivated *Bordetella pertussis* bacteria (Heimburg-Molinaro J. *et al.* 2009). The immunized mice produced T antigen reactive antibodies. I believe this study contributes further to the notion that immunological responses to T antigen can be elicited, and as inactivated bacterial were used in the vaccine formulation, this further supports the notion that specific danger signals are essential to T antigen priming.

The response observed to MUC1-T was however, only observed in mice living in a non-SPF environment, thus suggesting that in our murine model priming of cells required an educated immune system to perpetuate their immunological responses to this antigen. Many years ago it was reported that mice living in a SPF environment developed a less educated immune system compared to mice living in a non-SPF environment (Makinodan T. *et al.* 1971). Moreover, the involvement of microbiota

in immune responses has been illustrated in graft-versus-host disease (GVHD), a common complication following tissue transplantation that is particularly associated with hematopoietic stem cell transplantation (Heidt P. J. and Vossen J. M. 1992). When mice were treated with broad-spectrum antibiotics to eliminate aerobic and anaerobic bacteria in the gut, clinical symptoms of GVHD were completely eliminated and tissue pathology in the liver and colon reduced (Brandon J. A. *et al.* 2011). In addition, in a recently developed murine treosulfan-based allogeneic transplantation model for intestinal GVHD, it was shown that the induction of the disease was accompanied by a shift in gut flora towards pro-inflammatory bacterial species mediated by TLR9 signalling (Heimesaat M. M. *et al.* 2010). Thus it would be interesting to further explore the relationship between flora and the induction of MUC1-T immunological responses and TLR9 signalling.

Further evidence of the importance of microbiota in influencing the immune response to non-bacterial antigens is illustrated by the induction of oral tolerance in SPF mice but not in totally germ free mice (Ishikawa H. *et al.* 2008). The SPF mice had an increased systemic proportion of CD25<sup>+</sup> CD4<sup>+</sup> regulatory T cells (T<sub>reg</sub>) compared to germ-free mice, and these Tregs produce greater quantities of interleukin IL-10 and TGF- $\beta$ .

It has been shown in this thesis that MUC1-ST did not induce an immunological response in MUC1 Tg mice, or even in wild-type mice. However MUC1-ST-Fc, possibly through binding to APCs through their Fc receptors, has the potential of being processed by cellular components of the immune system. These results lead to the reasonable assumption that MUC1-ST may have immunoevasive and/or immunosuppressive properties.

Sialic acid on the non-reducing ends of oligosaccharides chains on the surface of cells has been shown to play a role in preventing cell-cell interactions through charge-repulsion (Kelm S. and Schauer R. 1997, Schauer R. *et al.* 1997). Thus, high sialic acid content found on the non-reducing ends of cleaved, serum MUC1 could, by charge-repulsion, inhibit binding and internalization by certain cellular components of the immune system such as DCs. In contrast to charge-repulsion, it is well documented that sialic acid binds immunoglobulins-like lectins (Siglecs), and

that sialic acids in an  $\alpha$ -2,3 linkage to Gal on MUC1 can bind Siglec-1 (Nath D. 2001), and Siglec-4 (Swanson B. J. *et al.* 2007). However, Siglec-1 does not contain an ITIM motif, and Siglec-4 is not expressed by haematopoietic cells. However, the vast majority of Siglecs do express an ITIM motif and are expressed mainly by haematopoietic cells (see introduction section 1.5.1). Furthermore, sialic acid in an  $\alpha$ -2,3 linkage is the preferred ligand for Siglecs-5, 9 and 10. Other Siglecs have, as their preferred ligand, sialic acid in an  $\alpha$ -2,6 linkage, and include Siglecs-2, 3 and 7, however this is only the preferred linkage and these Siglecs may bind sialic acid with other linkages.

On the whole, evidence appears to indicate that the primary function of Siglecs is to dampen host immune responses and Siglecs can be targeted by sialylated pathogens to evade the immune response (Cao H. and Crocker P. R. 2011, Jandus C. *et al.* 2011). Thus, a possible mechanism by which tumour cells evade immunosurveillance is through expression of high level of sialic acids on surface proteins such as MUC1, which would be able to dampen immune responses by engaging receptors of the Siglec family. Indeed, Mungul in 2004 proposed an immunosuppressive role for MUC1-ST (see section 1.11 on MUC1-ST and Mungul A. *et al.* 2004). Moreover, secreted cancer-associated MUC1, which has been shown to contain sialyl-T (Storr S. J. *et al.* 2008), has been associated with immunosuppression through inhibition of T-cell proliferation (Agrawal B. *et al.* 1998, Monti P. *et al.* 2004). However, at the moment the mechanism by which the cancer associated MUC1 and/or other surface/secreted proteins, high in sialic acid contents may dampen down possible immunological responses remains to be clarified. Currently the functional consequence between binding of recombinant MUC1-ST to various Siglecs is on-going in our laboratory (see section 6.5).

### **6.3 Development of human ST3Gal-I transgenic mice**

As discussed in the introduction (section 1.7) at least four mechanisms have been attributed to the promotion of the altered O-glycan profile in cancer; changes in expression of glycosyltransferases, changes in Golgi pH, changes in the location of glycosyltransferases and mutations in the *Cosmc* gene.

Cosmc loss of function/mutation has been found in neoplastic cell lines, including human colorectal carcinomas LSC, and in human cervical cancer (Ju T. *et al.* 2008). However, Cosmc loss of function/mutation does not appear to be a common event in breast cancer (Yoo N. J. *et al.* 2008, and personal data in appendix I). Alternatively, pH alteration is commonly associated with malignant transformation and growth (Yuan J. *et al.* 2000, Hill R. P. *et al.* 2001). Moreover, Golgi pH is known to be necessary for the optimal activity and correct localization of glycosyltransferases (Bretz R. *et al.* 1980, Varki A. 1998, Axelsson M. A. B. *et al.* 2001) and altered pH correlates with T-antigen expression in cancer cells derived from breast (Rivinoja A. *et al.* 2006). However, the major mechanism in breast cancer that results in aberrant O-linked glycosylation are changes in the expression of glycosyltransferases, and in particular the increase in ST3Gal-I expression. Furthermore, ST3Gal-I increased expression has been correlated with tumour grade (Burchell J. *et al.* 1999), and *in vitro* studies have shown that over-expression of ST3Gal-I is sufficient to cause the change from core 2 based glycans to sialyl core 1 glycans (sialyl-T) carried on mucins commonly found in breast cancer (Dalziel M. *et al.* 2001, Brockhausen I. 2006).

In this project, in order to increase the understanding of the relationship between ST3Gal-I overexpression and breast cancer, transgenic mice that overexpress ST3Gal-I in epithelial cells, including those of the mammary gland were generated. In order to have a model as close as possible to the human system, human ST3Gal-I cDNA was used. Crucially, a portion of the MUC1 promoter region was used to drive the expression of the ST3Gal-I transgene in order to selectively express the sialyltransferase in tissues where MUC1 is expressed, including the mammary glands.

Indeed, in our system and as reported by Graham and colleagues (Graham R. A. *et al.* 2001) the section of the MUC1 promoter used contained all the elements for expression of hST3Gal-I in mammary glands and in other tissues where MUC1 is expressed. The generated hST3Gal-I transgenic mice had no overt abnormal physical or behavioural phenotypes, and when crossed with MMTV-PyMT mice a unique

strain of mice that developed spontaneous tumours in the mammary glands was generated.

## **6.4 Involvement of ST3Gal-I in tumourigenesis**

Similarly to the way that Charles Darwin described evolutionary changes by natural selection, a way of looking at tumorigenesis is perhaps to think that functional changes that occur in tumour cells that result in a survival benefit, will be selected. As ST3Gal-I is found to be over-expressed in the majority of breast cancers it was hypothesized that this increase conferred an advantage to tumour cells. Therefore, as an increase in ST3Gal-I results in an increase in O-linked sialylated core 1 type glycans the resulting glycosylation should provide the tumour cells with a survival advantage, perhaps through dampening the immune response as discussed earlier.

In order to test if over-expression of ST3Gal-I did indeed confer an advantage to tumour cells, human ST3Gal-I Tg mice were crossed with MMTV-PyMT mice. Crossing of the hST3Gal-I Tg mice with PyMT mice clearly indicated, for the first time, that ST3Gal-I expression is beneficial to tumour cells as spontaneous tumours arose significantly earlier in these mice. The tumours did not show significant differences in terms of morphology or mitotic index, suggesting that ST3Gal-I expression did not affect the histopathological type of the tumour. Interestingly, a recent study conducted by Ma and colleagues, found that at least four distinct cellular populations can be recovered from PyMT tumours, and include two populations, one epithelial and one mesenchymal, which survive and proliferate *in vitro*. The epithelial population of cells studied by Ma, exhibited a strong tumour initiation potential needing as few as 10 cells to establish a tumour in syngeneic immune-competent mice suggesting that this population contains the cancer stem cells or cancer initiating cells (Ma J. *et al.* 2012). By definition of a tumour as defined by Hanahan (Hanahan D. and Weinberg R. A. 2011) these tumour cells may be able to evade the immune system even without having a fully developed tumour microenvironment. Interestingly, it has been reported by the Finn group that a population of cells within the total MCF-7 population have stem cell characteristics and express MUC1 carrying sialylated core 1 glycans (Engelmann K. *et al.* 2008).



Thus, perhaps tumour-initiating cells described by Ma and colleagues may be able to evade immune surveillance due to functionally advantageous changes resulting in the expression of sialyl-T. Tumour cells derived from hST3Gal-I/PyMT Tg mice may, from the onset, have the potential to limit immunological killing by expressing increased levels of sialylated core 1 on proteins. Indeed in the eye, the limbal stem cells do not express  $\alpha$ -2,3 sialylation but the initial differentiation involves sialylation of core 1 and the loss of expression of peanut agglutinin (PNA) binding (Wolosin J. M. and Wang Y. 1995). These sialyl T expressing cells were found to be highly proliferative as compared to the limbal stem cells, and their surface glycosylation was associated with proliferative properties (Wolosin J. M. and Wang Y. 1995). In our model, by the time the tumour reached the palpable size required to start measurements the total  $\alpha$ -2,3 sialylation on tumour cells derived from the two groups was found to be similar as detected by mass spectrometry. However, it is possible that the tumour initiating cells within these mammary tumours in PyMT mouse model only start to be highly proliferative when the cells acquire an increase in  $\alpha$ -2,3 sialylation. This could potentially explain the significant earlier appearance of the tumours in the mice overexpressing ST3Gal-I.

## **6.5 Strategic plan / further investigations**

Data included in this thesis contribute to the understanding of how MUC1 carrying specific and defined O-linked glycans can be expressed and purified using wild-type CHO cells and mutant CHO cells. CHO cells were well suited for the production of the MUC1 glycoproteins for many reasons including the fact that the cells are widely available, fast growing, easily transfectable, can adapt well to growth in monolayer or in suspension and have been grown successfully in a variety of production systems, and very importantly do not express the core 2 glycosyltransferase (Bierhuizen M. F. A. and Fukuda M. 1992) and so the O-linked glycans carried by proteins expressed in these cells are core 1 based. The MUC1 glycoform produced in WT CHO cells is mainly sialylated core 1, one of the dominant cancer-associated glycoforms of MUC1 (Backstrom M. *et al.* 2003). However, it would be interesting to evaluate the production of MUC1 glycoproteins in human cell lines such as Hek 293, Per-C6 and CAP cells, which are emerging as powerful alternatives for the

production of human therapeutic proteins. These cell lines are easily grown in serum-free suspension cultures (Swiech K. *et al.* 2012), and would only incorporate Neu5Ac (when grown in serum free media) as humans have lost the ability to synthesize Neu5Gc (Deng L. *et al.* 2013). However, it is debatable if this would be an advantage as when using MUC1 glycoforms as immunogen to induce antibodies, MUC1 carrying Neu5Gc maybe desirable, as tumours appear to accumulate more of this sialic acid variant which is available from the diet. Moreover, Hek 293 cells can make core 2 based glycans (Brautigam J. *et al.* 2013); therefore expression of MUC1 in these cells may result in the secretion of MUC1 carrying core 2 glycans rather than the tumour-associated core 1. Interestingly, a recent study has shown that anti-apoptotic agents such as N-acetylcysteine and thiolactic acid can enhance glycoprotein production in CHO cells (Chang K. H. *et al.* 2013). This high production of glycoproteins can however result in a decrease in sialylation, but this could be compensated in the case of the production of MUC1-ST by the transfection of ST3Gal-I into the cells. Thus, I believe that given the right resources further optimization of the production system, perhaps using anti-apoptotic agents during the culture, could be achieved for the large-scale production of MUC1 glycoproteins for use as potential therapeutics.

The observation that the immune response to MUC1-T was lost when the MUC1 transgenic mice were rederived into a SPF unit suggests that the environment affected their immunological predisposition perhaps by influencing their indigenous microbiota. Indeed, as discussed under section 6.2 above, this is reminiscent of the lack of graft versus host disease observed in mice lacking gut flora. Although the T cell response to MUC1-T was measured in the SPF mice, due to time constraints the presence of circulating antibodies was not measured nor was the secretion of Th2 type cytokines. Thus we are unable to say if a humoral immune response could be generated in these SPF mice. Future studies should evaluate the levels of MUC1 antibodies in the sera of SPF immunised mice and the presence of Th2 cytokines.

Interestingly, the experimental MUC1 transgenic mice living in a dirty environment were found to carry *Helicobacter pylori* (*H. pylori*), a bacterium linked to the increase in antibodies to MUC1 (Klamas K. *et al.* 2007) and T antigen (Klamas K. *et al.* 2002).

Thus, there appears to be a link between the development of T cell responses and microbial flora, and a number of experiments could be performed in order to further investigate this link:

- 1) A detailed analysis of the immune system of MUC1 transgenic mice living in a “dirty” environment or a SPF environment could be performed. This would include measurement of circulation cytokines including IL-4, IL-5, IL-6, IL-10, IL-12, TNF $\alpha$  and TGF $\beta$ , and the frequencies of CD25<sup>+</sup>, CD4<sup>+</sup> Tregs in the mesenteric lymph nodes.
- 2) By infecting mice living in an SPF environment with *H. pylori* and/or other bacteria in a controlled manner it may be possible to assess the extent by which indigenous microbiota may contribute to the induction of an immune response to MUC1-T. Moreover, perhaps probiotic adjuvants such as *Lactobacillus* (e.g. *L-reuteri* ATCC no 23272) which is suggested to play an important role in modulating immunological function by promoting Th1 responses (Mohamadzadeh M. *et al.* 2005) could be included with the MUC1-T immunizing agent.

If an immune response could be generated by adding back bacterial flora, the investigations into the use of MUC1-T as a cancer vaccine could continue. The efficacy of MUC1-T vaccination in preventing tumour formation in a prophylactic and therapeutic setting should be tested. In a transplantable tumour setting the murine tumour cell lines RMA or MC38 expressing human MUC1 could be used. The presence of MUC1 carrying T on these lines can easily be established by ELISAs using HMFG2 to pull down MUC1 followed by PNA detection before and after sialidase treatment. The presence of MUC1-T could be confirmed by the binding of the MUC1-T specific monoclonal antibody 1B9 (Tarp M. A. *et al.* 2007). However, the necessity of MUC1-T expression by the tumour cells is unclear as the T glycan may be the means by which the MUC1 is taken up by the antigen presenting cell to allow processing and presentation on MHC class II to CD4 (see chapter 4). Engineering the glycan expression by transfecting or knocking-down the relevant glycosyltransferases in the tumour cell line could answer this question. Furthermore, the use of an *in vivo* system such as the one that spontaneously develops mammary tumours (MMTV-PyMT) crossed with MUC1-Tg mice (MMTV-PyMT/MUC1) could be used to test the potential of MUC1-T as a true

vaccine (immunization before tumour development). These experiments would give important preclinical data and could form the basis of work aimed at an initial phase I clinical trial.

Work carried out in this thesis using recombinant MUC1-ST (Neu5Ac $\alpha$ 2,3Gal $\beta$ 1,3GalNAc) suggests that this particular glycan carried on MUC1 may have immunosuppressive properties. Indeed, MUC1 has already been associated with immunosuppression through inhibition of T-cell proliferation or inhibition of the maturation of dendritic cells (Agrawal B. *et al.* 1998, Rughetti A. *et al.* 2005), and our investigation appears to have defined the glycoform responsible. A number of further studies could be initiated to confirm the immunosuppressive properties of recombinant MUC1-ST and these include:

- 1) Immunizing wild-type mice with MUC1-ST in conjunction with immunogenic proteins such as recombinant MUC1-T, Tn or unglycosylated MUC1. A preliminary experiment has already been attempted by immunizing mice with ovalbumin in the presence of MUC1-ST. However, in this instance MUC1-ST did not abolish the T cell response to SIINFEKL, but as this is an extremely strong immunogen, perhaps this is not too surprising.
- 2) Investigating the potential interactions of MUC1-ST with Siglecs. The preferred ligand for many Siglecs is the  $\alpha$ -2,3-linked sialic acid, and Siglecs are involved in dampening down the immune response to self, suggesting a mechanism whereby MUC1-ST could inhibit immune responses. Previous studies have shown that MUC1 can bind Siglec-1 (Nath D. 2001) and our laboratory also found that recombinant MUC1-ST binds to Siglec-1 (unpublished data). Unlike most other Siglecs, Siglec-1 does not have an immunoreceptor tyrosine-based inhibitory motif (ITIM) and it has been suggested to act as a scavenger receptor removing sialylated molecules from the system. Thus, it would be interesting to investigate recombinant MUC1-ST immunization in Siglec-1 KO and Siglec-1 KO/MUC1 TG mice.

The data presented in this thesis clearly show for the first time in an *in vivo* model, that increased ST3Gal-I levels in epithelial tissues, including the mammary gland, promotes mammary gland carcinogenesis, as tumours arose significantly earlier in

hST3Gal-I/PyMT mice than in control mice. However, at present the mechanism by which ST3Gal-1 promotes carcinogenesis is unclear. It is apparent that by the time samples were taken for analysis any acquired tumorigenic advantage in hST3Gal-I/PyMT as compared to the control mice, would have been mitigated. Thus in order to further explore the role of ST3Gal-1 in tumorigenesis, it will be necessary to investigate early tumorigenic events in hST3Gal-I/PyMT mice as compared to PyMT control mice.

Several mechanisms could be responsible for promoting tumorigenesis, including the ability for the tumour to escape immune surveillance early in tumour development and indeed data from a transplantable tumour model suggest that this may play an important role (Mungul A. *et al.* 2004). In order to address this the number of infiltrating lymphocytes including NK, CD4, CD8 and Treg and the level of expression of cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$ , IL-2, IL-4, IL-5 and IL-10 in hyperplastic mammary glands in hST3Gal-I/PyMT mice as compared to the PyMT control mice could be measured in sections of the gland.

Many of the pathways activated in the PyMT tumours are those that are also activated in human cancers (Al Masri M. and Gendler S. J. 2005). Therefore, further experiments could determine which, if any, of these pathways are perturbed in the ST3Gal-1/PyMT mammary glands at the early stage of tumour development. As Muc1 has been shown to play a role in the tumorigenesis of PyMT, initial investigations could involve the pathways that this mucin has been shown to play a role in (Al Masri M. and Gendler S. J. 2005). This could involve looking at the:

- Interaction of  $\beta$ -catenin and c-Src in hyperplastic glands from ST3Gal-I tumours and controls.
- Interaction of cSrc with PI3Kinase and the phosphorylation status of AKT in hyperplastic glands.
- The phosphorylation status of Stat3.
- The association of Muc1 with FAK.

## 6.6 *Final remarks*

Data included in this thesis contributes to the understanding of how MUC1 carrying specific and defined O-linked glycans can be expressed and purified using CHO cells and CHO derivatives systems. Furthermore, different O-linked cancer-associated MUC1 glycoforms are found to have different immunological properties. This is important as if MUC1 is to be used in any immunotherapeutic strategies the data presented here show that it is of vital importance to select a specific and immune activating glycoform. Evidence is now mounting that the MUC1-ST glycoform may have immunosuppressive effects or at the very least is non-immunogenic. In addition, we have demonstrated that the high expression of the glycosyltransferase that adds sialic acid to core 1 to form ST results in a significantly decreased time to tumour presentation in a murine model of spontaneous mammary tumours. This is the first demonstration that an enzyme involved in O-linked glycosylation can promote tumorigenesis. However, the mechanism involved remains elusive, although previous data (Mungul A. *et al.* 2004) and our studies on the immune response of specific MUC1 glycoforms suggest it may be immunologically related.

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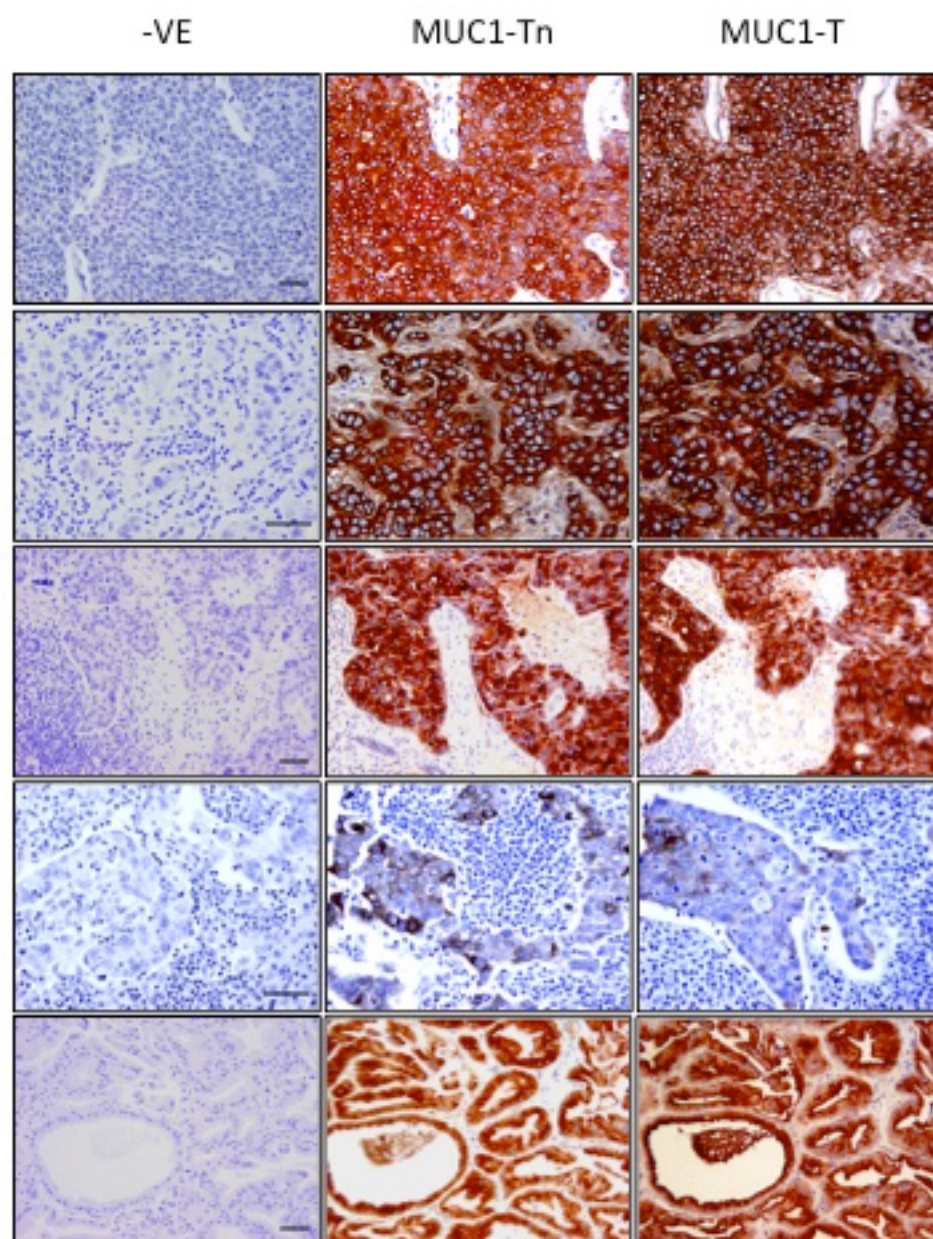
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## Appendix I



### *Expression of MUC1-Tn and MUC1-T in primary breast carcinomas.*

Serial formalin fixed paraffin embedded sections from primary breast carcinomas was stained with the 5E5 and IB9 monoclonal antibodies. The monoclonal 5E5 recognises Tn, and to a lesser extent STn, but only when the glycan is carried on a MUC1 backbone (Sorensen A. L. *et al.* 2006, Tarp M. A. *et al.* 2007). IB9 recognises the T glycan only when carried by MUC1 (Tarp M. A. *et al.* 2007).

## *Appendix II*

### *EU consortium*

#### Participants

Partner 1	Co-ordinators	J Taylor-Papadimitriou & J Burchell
Partner 2	Copenhagen	H Clausen
Partner 3	Goteborg	G Hansson
Partner 4	Heidelberg	T Nilsson
Partner 5	Juelich	T Noll
Partner 6	Rome	M Nuti
Partner 7	Umea	J Kihlberg
Partner 8	Zurich	K Moelling/J Pavlovic
Partner 9	ICRT	D Snary

*Dr Henrik Clausen*  
*School of Dentistry*  
*Dept of Diagnostics*  
*University of Copenhagen*  
*Norre Alle 20*  
*DK 2200 Copenhagen N*  
*DENMARK*  
*E: [henrik.clausen@odont.ku.dk](mailto:henrik.clausen@odont.ku.dk)*

*Dr Gunnar Hansson*  
*University of Goteborg*  
*Dept of Medical Biochemistry*  
*Medicinargatan 9*  
*41390 Gothenburg*  
*SWEDEN*  
*E: [gunnar.hansson@medkem.gu.se](mailto:gunnar.hansson@medkem.gu.se)*

*Dr Tommy Nilsson*  
*Cell Biology & Biophysics Programme*  
*Meyerhofstrasse 1*  
*D-69017 Heidelberg*  
*GERMANY*  
*E: [nilsson@embl-heidelberg.de](mailto:nilsson@embl-heidelberg.de)*

*Dr Thomas Noll*  
*Head of Cell Culture*  
*Technology Group*  
*Institute of Biotechnology 2*  
*Forschungszentrum Julich GmbH*  
*52425 Julich*  
*GERMANY*  
*E: [th.noll@fz-juelich.de](mailto:th.noll@fz-juelich.de)*

*Dr Marianna Nuti*  
*Dipartimento di Medicina*  
*Sperimentale e Patologia*  
*Viale Regina Elena 324*  
*Roma 00161*  
*ITALY*  
*E: [marianna.nuti@uniroma1.it](mailto:marianna.nuti@uniroma1.it)*

*Prof Jan Kihlberg*  
*Organic Chemistry*  
*Umea University*  
*S-90187 Umea*  
*SWEDEN*  
*E: [jan.kihlberg@chem.umu.se](mailto:jan.kihlberg@chem.umu.se)*

*Prof Karin Moelling*  
*Dr Jovan Pavlovic*  
*Director of the Institute of Medical*  
*Virology*  
*Institute of Medical Virology*  
*University of Zurich*  
*Zurich*  
*SWITZERLAND*  
*E: [moelling@immv.unizh.ch](mailto:moelling@immv.unizh.ch)*  
*E: [pavlovic@immv.unizh.ch](mailto:pavlovic@immv.unizh.ch)*

*Dr David Snary*  
*Applied Development Laboratory*  
*Imperial Cancer Research Technology*  
*Dominion House*  
*59 Bartholomew Close*  
*LONDON*  
*EC1A 7BE*  
*E: [d.snary@icrf.icnet.uk](mailto:d.snary@icrf.icnet.uk)*

### *Appendix III*

#### *List of publication during the thesis*

Picco G, Julien S, Brockhausen I, Beatson R, Antonopoulos A, Haslam S, Mandel U, Dell A, Pinder S, Taylor-Papadimitriou J, Burchell J. 2010. Over-expression of ST3Gal-I promotes mammary tumorigenesis. *Glycobiology* 20: 1241-50

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## Appendix IV

### *Human ST3Gal-I sequence (L29555)*

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gaggaggagg aagaagagcg gagagagaag gaagaggcga tgtgagctgg
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